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DEFINITIVE HOST RELATIONSHIPS OF THE HELMINTH PARASITES OF THE DEER MOUSE, *PEROMYSCUS MANICULATUS*, IN THE BONNEVILLE BASIN OF UTAH*

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The parasitism of the deer mouse, *Peromyscus maniculatus*, was investigated as a phase in the study of parasitism in isolated rodent populations inhabiting the "island" or desert mountain ranges in and bordering the Bonneville Basin. The study area is comprised of a 20,000-square-mile area composing the northern half of the basin occupied by ancient Lake Bonneville and now occupied in part by the Great Salt Lake and the Great Salt Lake Desert. All of the mountain masses involved, except the Wasatch, are isolated from each other by considerable distances of desert valley and salt flats, a type of habitat that effectively isolates populations of montane rodent species to the habitats of the higher mountains they currently occupy. The mountain ranges of the area included in the study are the Deep Creek, Raft River, Stansbury, Wasatch, Oquirrh, Onaqui, Cedar and Fish Springs.

Ecological habitat in the region is varied and extends from the barren salt flats, where no vegetation grows, to the alpine tundra above timberline in the Wasatch and Deep Creek Mountains. In ascending the mountains from the valley bottoms, one passes successively through (1) a pickleweed community bordering the salt flats and lake, (2) a desert valley shadscale-greasewood association, (3) a mixed brush of shadscale and horsebrush bordering the mountains, (4) the lower mountain slopes of pigmy forest of juniper-sage brush, (5) the brush of the higher and drier mountain slopes and canyon walls, (6) the fir-spruce-aspen representing true montane conditions, and (7) an alpine tundra. A distinct streamside association occurs that changes in character as one passes to higher elevations. In some locations stabilized dunes with varied vegetation occur in dry foothills and valley bottoms.

The rodent species of the area vary in their ability to occupy the diverse habitats present. Some forms (such as the subspecies of *Thomomys talpoides* and *T. umbrinus*, the pocket gophers of the region; the chipmunks, *Eutamias dorsalis* and *E. amoenus amoenus* (Allen); the long-tailed pocket mouse, *Perognathus formosus incolatus* Hall; and the Great Basin pocket mouse, *P. parvus olivaceus* Merriam) are restricted to suitable habitats in the mountains and the populations, as now constituted, are considered to have been isolated with little gene interchange for the thousands of years since Lake Bonneville occupied the basin. The two subspecies of *Peromyscus maniculatus* present in the area inhabit the entire region and are present from the pickleweed to the alpine tundra. *Peromyscus* populations of the desert and desert mountains other than the Wasatch Mountains have been referred by Durrant (1952) to *P. maniculatus sonoriensis* (Le Conte). The higher Wasatch Mountains are inhabited by *P. m. rufinus* (Merriam) which interbreeds freely in the foothill region with *sonoriensis* producing a broad belt of intergradation with the specimens

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at the lower edges referable to typical *sonoriensis* and those at the upper border closely resembling *rufinus*.

DISCUSSION

In attempting to evaluate data obtained in the study of parasitism in isolated rodent populations, it was concluded that no accurate assessment could be made of the effects, or the extent, of isolation without a knowledge of the non-host-specific parasites present both in the isolated rodent species and in the widely distributed rodent species, the ranges of which coincided or overlapped. *P. maniculatus* was given special attention because of its universal distribution in all habitats, its high population density, and the fact that it is host to more of the parasitic species infecting other rodents than other wide ranging mammals of the area such as the desert jack rabbit, *Lepus californicus deserticola* Mearns. Also, from data collected over a 5-year period, it has been observed that host specific parasites generally tend to be unevenly distributed and seem to require more precise habitat conditions which preclude general distribution over the region. Incidence of infection with host specific forms may be low, but was observed to be very high in some localities. *Syphacia peromysci* Harkema, 1936, which is almost specific for *Peromyscus* species, is an exception in that it appears to be universally distributed and was present in all localities collected. Non-host-specific forms were widely and more evenly distributed and usually showed a reasonably high incidence of infection. As can be noted in table II, the non-host-specific parasites have a wide distribution throughout a region with diverse habitat indicating that the lack of host specificity is probably a greater asset in the variable desert-mountain type of habitat as found in the Bonneville Basin than in extensive areas of a single biome.

Work, involving parasitism in deer mice, previously completed in the region is that of the author (Grundmann 1957, 1958) on the nematodes and cestodes of the Great Salt Lake Desert. The current study has materially increased the list of parasites by extending the study into the mountains surrounding the desert area.

Many of the forms collected from local deer mice have been reported previously in *Peromyscus* species of other regions of the United States and Canada. Hall (1916) examined a collection of nematodes from the museum at Colorado College and described as new a number of species present in the Colorado region, of which, several were subsequently collected in the present study. Other studies have been made by Harkema (1936), Erickson (1938), Lubinsky (1957), and Voge (1956). Kruidenier and Peebles (1958) have contributed records from the Grand Canyon of Arizona, and Smith (1951, 1954) reported on parasites obtained from the dissection of numerous small mammal specimens from the high plains east and south of the Rocky Mountains.

RESULTS AND CONCLUSIONS

Table I is composed of a list of the parasitic species recovered from *P. maniculatus* in the study area and a list of the other rodent species also found to be infected with these organisms. Blood and intestinal protozoa are not included as these are being reported separately. The seven species marked with an asterisk were found to be most widely distributed and were collected from almost all localities visited. Nine species were found to be present in rodents other than *P. maniculatus*. It was

TABLE I. *Parasitic species present in* Peromyscus maniculatus sonoriensis *and* P.m. rufinus *in the Bonneville Basin with a list of other rodent hosts found infected.*

Parasite	Other rodent species infected
TREMATODA :	
Brachylaime microti Kruidenier and Gallicchio 1959	
CESTODA :	
Mesocestoides carnivoricolus Grundmann 1956 (larval)	*Peromyscus crinitus pergracilis* Goldman
**Catenotaenia linsdalei* McIntosh 1941	*Perognathus formosus incolatus* Hall
**Hymenolepis citelli* McLeod 1933	*Citellus leucurus leucurus* (Merriam)
	Thomomys talpoides wasatchensis Durrant
	Onychomys leucogaster utahensis Goldman
Cladotaenia circi Yamaguti 1935 (larval)	
Paruterina candelabraria (Goeze, 1782)	*Neotoma lepida lepida* Thomas
NEMATODA :	
Trichuris perognatha Chandler 1945	*Perognathus formosus incolatus* Hall
Nippostrongylus muris Yokagawa 1921	
**Syphacia peromysci* Harkema 1936	*Rheithrodontomys megalotis* (Baird)
***Aspiculuris tetraptera* Schultz 1924	
**Protospirura numidica* Seurat 1914	*Peromyscus crinitus pergracilis* Goldman
	P. truei nevadensis Hall and Hoffmeister
	Onychomys leucogaster utahensis Goldman
	Rheithrodontomys megalotis (Baird)
	Dipodomys ordii utahensis (Merriam)
**Rictularia coloradensis* Hall 1916	*Citellus leucurus* (Merriam)
	Eutamias minimus pictus (Allen)
	E. M. consobrinus (Allen)
	E. amoenus amoenus (Allen)
	Perognathus parvus olivaceus
ACANTHOCEPHALA :	
**Moniliformis clarki* (Ward 1917)	*Thomomys umbrinus stansburyi* Durrant
INSECTA :	
Diptera	
Cuterebra americana complex	

* Widely distributed species.
** Found only at Dugway Proving Grounds.

the data on host interrelationships of this latter group that constitutes the evidence from which conclusions were drawn concerning the role played by the deer mouse in the distribution of non-host specific forms to isolated or restricted populations of other rodent species.

Dispersal of some parasites of deer mice may be attributed to other agents. Three cestode species, *Mesocestoides carnivoricolus* Grundmann, 1956, *Cladotaenia circi* Yamaguti, 1935, and *Paruterina candelabraria* (Goeze, 1782), had a sporadic distribution and were obtained only as larval forms. The carnivorous mammal and bird definitive hosts are the means of dispersal for these forms. Dipteran larvae of the *Cuterebra americana* complex were found occasionally beneath the skin of deer mice. The adult fly is the agent of distribution, and from the frequency of occurrence, it is assumed that the deer mouse may not be the major host of the fly. *Trichuris perognatha* Chandler, 1945, has been previously found infecting *Perognathus formosus incolatus* Hall in the same habitat from which infected deer mice were collected in rare instances and the pocket mouse is considered the major host of the worm. Frequently, the lungs of deer mice and other rodent species were found with areas of cellular infiltration containing the spherical bodies of a species of *Haplosporangium*. Flukes of *Brachylaime microti* Kruidenier and Gallicchio were recovered from the bile ducts of deer mice collected from several locations in the Deep Creek Mountains which are located on the Utah-Nevada line.

Table II shows the habitat distribution of the six most abundant and widely distributed parasites found in *P. maniculatus* and their occurrence in the other host rodent species listed in table I. It will be noted that in all cases, except in *Hymenolepis citelli* McLeod 1933, the distributional range of the parasite in *P. maniculatus*

exceeds that observed in other rodents with more restricted habitat range.

Hymenolepis citelli was found to be a common parasite of ground squirrels, especially of *Citellus leucurus leucurus* Merriam, and subspecies of the pocket gophers, *Thomomys umbrinus* and *T. talpoides*. It was collected from these animals in habitats extending from the shadscale-greasewood to the conifer-aspen belts on the desert mountains. The tapeworm was present in deer mice only in the mountain brush and conifer-aspen habitats of the higher slopes and was absent from numerous specimens trapped from desert situations on the lower mountain slopes and in the valleys. The omnivorous diet and changing food habits of deer mice in the variety of habitat conditions occupied by them explains the patterns of infection observed.

Parasitic species exhibiting a high incidence of infection and a general distribution over the area also show great tolerance to diverse climatic conditions and lack of host specificity. Those forms having a direct life cycle have eggs resistant to extremes of temperature and moisture. *Syphacia peromysci* is presumably of this type. Parasites using arthropods as intermediate hosts, such as *Protospirura numidica, Rictularia coloradensis* and *Moniliformis clarki,* must either occupy a number of arthropod species in the various habitats or several that have as broad a range as the deer mouse.

The following are conclusions drawn from the data assembled through the dissection of over 1,200 animals of all species listed in table II: (1) The deer mouse, with its almost universal distribution and relatively high population density, has distributed the parasitic species to which it is victim to all ecological habitats of the

TABLE II. *Habitat distribution of 6 common parasitic species of the deer mouse showing habitat distribution in other rodent hosts infected.*

Parasite	Pickle-weed	Valley bottom shadscale-greasewood	Valley slope. Mixed brush. Horse-brush-shad-scale-black sage	Pigmy forest Juniper-sage brush	Mountain slopes and canyon walls brush habitat	Hud-sonian conifer-aspen
CESTODA:						
Catenotaenia linsdalei		x	x	(6) x		x
Hymenolepis citelli		(8)	(8)	(8) (11) (12)	x (8) (9) (11) (12)	x (9)
NEMATODA:						
Syphacia peromysci	x	x	x	x (5)	x (5)	x
Protospirura numidica		x (3) (4)	x (3) (4)	x (5) (1) (2) (3) (4)	x (1) (3) (5)	x
Rictularia coloradensis		x (1) (8) (11)	x (8) (11)	x (8) (11)	x (7) (8)	x
ACANTHOCEPHALA:						
Moniliformis clarki		x	x	x	x (10)	x

Distribution in *P. maniculatus* indicated by the letter x. Rodent hosts of the above parasites are designated by the following numbers:

Peromyscus crinitus pergracilis	(1)	*Perognathus parvus olivaceus*	(7)
P. truei nevadensis	(2)	*Citellus leucurus leucurus*	(8)
Onychomys leucogaster utahensis	(3)	*Thomomys talpoides wasatchensis*	(9)
Dipodomys ordii utahensis	(4)	*T. umbrinus stansburyi*	(10)
Rheithrodontomys megalotis	(5)	*Eutamias minimus pictus*	(11)
Perognathus formosus incolatus	(6)	*E. amoenus amoenus*	(12)

region which offer the necessary conditions to complete the life histories and has maintained the infections more or less permanently by seeding the region with ova from which other rodent species ultimately receive their infections. (2) The deer mouse may in turn also be infected in some localities with the normal parasites of other rodent species to which it is susceptible and carry these to other localities where the normal host population has been free of the parasite. (3) Populations of rodent species now restricted to mountain conditions, habitats in the area, and isolated from each other by desert, have been continually exposed to reinfection with parasites common to both *Peromyscus* and the isolated species. Thus at the present time, infections with these parasites were found to occur in isolated rodent populations of all mountain ranges studied. In this regard however, one must always recognize the ancient origin of most parasite species and include the possibility that the original populations at the time of isolation possessed these forms and have subsequently maintained them. (4) In order to determine the effects of isolation on parasite distribution and on parasitic species, one must know the host interrelationships of all species present so as to separate those that are host specific from those that are not. (5) Only parasitic species that are host specific can be used to study the effects of isolation.

SUMMARY

The deer mouse, with its high population and universal distribution, must be considered responsible for distributing its parasites to all habitats and localities, and where parasitic species are not host specific, of responsibility for maintaining and spreading them to isolated and restricted populations of other susceptible rodent species.

No accurate assessment can be made of the effects of isolation on parasites without a knowledge of the host-interrelationships of all animal species present in the region.

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RESEARCH NOTE

THE PARASITES OF SOME AMPHIBIANS OF UTAH.

During the summer of 1955, a survey was conducted of the parasites of the amphibians of Salt Lake County, Utah. During the course of this study, members of the following species of parasites were found in the hosts indicated:

Species of Parasite	*Species of Host*
(HAEMOPARASITIC) *PROTOZOA*	
Cytamoeba bacterifera	*Ambystoma tigrinum* (Green, 1825)
	Rana pipiens Schreber, 1872
(INTESTINAL) *PROTOZOA*	
Opalina carolinensis Metcalf, 1923	*Bufo boreas* Baird and Girard, 1852
	Rana pipiens
Nyctotherus cordiformis Ehrenberg, 1838	*Bufo boreas*
	Rana pipiens
Chilomastix caulleryi Alexeieff, 1909	*Ambystoma tigrinum*
	Bufo boreas
Hexamastix batrachorum Alexeieff, 1911	*Bufo boreas*
	Rana pipiens
Hexamastix sp. (large)	*Ambystoma tigrinum*
Hexamastix sp. (small)	*Rana pipiens*
Hexamita Dujardin, 1841	*Ambystoma tigrinum*
	Bufo boreas
	B. woodhousii Girard, 1854
	Rana pipiens
Karotomorpha swezyi (Grassé, 1926)	*Ambystoma tigrinum*
Octomitus pulcher (Becker, 1926)	*Bufo boreas*
Proteromonas longifila (Lemmermann, 1913)	*Bufo boreas*
	Rana pipiens
Tritrichomonas augusta (Alexeieff, 1911)	*Ambystoma tigrinum*
	Bufo boreas
	B. woodhousii
	Rana pipiens
T. batrachorum Perty, 1852	*Ambystoma tigrinum*
Tritrichomonas sp. indet.	*Rana pipiens*
CESTODA	
Ophiotaenia filaroides LaRue, 1909 (adults and plerocercoids)	*Ambystoma tigrinum*
TREMATODA	
Halipegus Looss, 1899	*Rana pipiens*
Haematoloechus coloradensis Cort, 1915	*Bufo woodhousii*
	Rana pipiens
Phyllodistomum bufonis Frandsen, 1957	*Bufo boreas*
Alaria Schrank, 1788 (mesocercariae)	*Bufo woodhousii*
	Rana pipiens
NEMATODA	
Cosmocercoides dukae (Holl, 1928) Travassos, 1931	*Bufo boreas*
Oxysomatium giganticum (Olsen, 1938)	*Bufo boreas*
Spironoura elongata (Baird, 1858) Walton, 1932	*Ambystoma tigrinum*

Relatively little work has previously been done on the parasites of the amphibians of the Lake Bonneville Basin. The parasites listed above were collected from a large number of localities, ranging from the lowlands adjacent to the Great Salt Lake to lakes high in the Wasatch Mountains. The authors are indebted to Dr. Frederick R. Evans for aid in identification of the intestinal protozoa.—JOHN C. FRANDSEN AND ALBERT W. GRUNDMANN, *Department of Zoology and Entomology, University of Utah, Salt Lake City.*

THE ECOLOGY OF THE FISH *GILLICHTHYS MIRABILIS* AND ONE OF ITS NEMATODE PARASITES

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In July 1957 we began a three-year ecological study of the fish *Gillichthys mirabilis* Cooper (the mud sucker or long-jawed Goby) and its metazoan and protozoan parasites. We had become convinced that before a complete picture of a host and its parasites can be described, an investigation of all of the parasites in many hosts, examined at least monthly over a period of a year or more, must be made because every species of parasite in or on a host body is part of the effective environment of every other species belonging to that host. As many ecological factors as possible, therefore, are being studied.

From weekly collections of fish during a period of 24 months we have found 15 species of parasites. The species which occurs in greater numbers and which invades more hosts than all the others is a nematode of the genus *Spirocamallanus* Olsen. This worm is, in a sense, our key parasite, and we wish to redescribe it because existing descriptions in the literature are either incomplete or they are not in agreement with each other.

The morphological description of the nematode will be accompanied by pertinent ecological observations, and succeeding papers will attempt to complete the account of the ecology of *Gillichthys mirabilis* and its parasite-mix. This study was made with the aid of a grant from the National Science Foundation.

Review of the Literature on Spirocamallanus pereirai (*Annereaux*) *Olsen*

Procamallanus spiralis was described from a single male specimen by Baylis (1923a) from the Silurid fish *Heterobranchus anguillaris* from the Nile River at Cairo. Later in the same year Baylis (1923b) expanded the description by adding detailed measurements of the female, but certain details relating to the caudal papillae of the male were lacking. In 1931 Törnquist redescribed the species, cited new host references (see table I) and added details on morphological and cytological features of both male and female worms. In 1946 Annereaux described a new species, *P. pereirai*, and he developed a key for the 22 existing species of the genus *Procamallanus*. His paper included the first report of the genus in North America. The next paper concerned with this worm was by Olsen (1952), who divided the genus into *Procamallanus* and *Spirocamallanus*. He also described a new species which he placed in the new genus *Spirocamallanus*.. The genus *Procamallanus* was restricted to those eight species having a smooth buccal capsule lining. The new genus was characterized by spiral thickenings on the inner wall of the buccal capsule. Sixteen species were referred to the new genus. In 1953 Winter reviewed the literature on these nematodes, and redescribed *Spirocamallanus spiralis*. He worked on parasites from Mexico, and based his account on females only. In 1955 Sogandares-Bernal established a new host record for *S. pereirai* from Louisiana. Finally,

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Ali (1956) in India modified the key developed by Annereaux, and he described five new species of *Procamallanus,* three of which probably would have been placed in the genus *Spirocamallanus* had Ali noted the paper by Olsen. Ali developed a key for the identification of species of the original genus *Procamallanus,* but the key is based on certain measurements which do not correspond with some of those previously reported.

The present information on the distribution of *Spirocamallanus pereirai* and the closely related *S. spiralis* is summarized in table I.

MATERIALS AND METHODS

The material for this study was taken from fish found in the Goleta Slough, a tidal estuary situated approximately 8 miles west of the city of Santa Barbara, California. This slough is fed daily by incoming tides and likewise drained. It is extensive in area, and it serves as the drainage basin for the eight small creeks that flow into it through the Goleta Valley. This movement of water ensures a marked fluctuation in its salinity. During the winter rains the mouth of the slough is occasionally closed by a heavy deposition of sand which causes the water to remain fresh, or nearly fresh, for weeks at a time.

During the period from July 1957 to July 1959, 810 fish were caught in wire-mesh, cylindrical minnow traps (with a funnel at each end) which were placed at various locations in the slough. The fish were taken from the traps and brought to the adjacent laboratory and examined immediately, or at least within 24 hours, for their parasite fauna.

The nematode parasites were taken alive from the intestines of the fish and fixed in warm 70 percent ethyl alcohol. The unstained specimens were usually cleared in glycerol and mounted either in glychrogel or poly-vinyl alcohol.

TABLE I. *Distribution and host record of* Spirocamallanus pereirai *and* S. spiralis.

Author	Species of *Spirocamallanus*	Host	Location	Numbers of parasites used for description
Baylis 1923a	*spiralis*	*Heterobranchus anguillaris*	Cairo	one male
Baylis 1923b	*spiralis*	*Synodontis cupterus*	Khartoum	several males and 3 females
Törnquist 1931	*spiralis*	*Crenidens forskalli* *Sargus noct* *Cheilinus trilobatus*	Gulf of Suez	8 females 4 males
Annereaux 1946	*pereirai*	*Atherinopsis californiensis*	Bolinas Bay, California	one male 3 females
Olsen 1952	Divided genus into *Procamallanus* and *Spirocamallanus.*			
Winter 1953	*spiralis*	*Micropogan ectenes* *Achirus* sp.	Vera Cruz River, Oaxaca, Mexico	8 females
Sogandares-Bernal 1955	*pereirai*	*Micropogan undulatus*	Lake Pontchartrain, Louisiana	numerous
Ali 1956	Developed a new key for the original genus *Procamallanus.*			

DESCRIPTION OF THE PARASITE

The original description of *Spirocamallanus pereirai* by Annereaux (1946) gave the following characteristics: "Body slender; attenuated towards both extremities. Anterior end truncated; oral opening rounded, terminal. Buccal capsule barrel-shaped and provided with 14 uninterrupted spiral thickenings in both sexes. Cuticle thick, and finely striated transversely in cephalic region; striations much coarser in posterior end." See figure 1 for the buccal capsule. Specimens in life reddish in

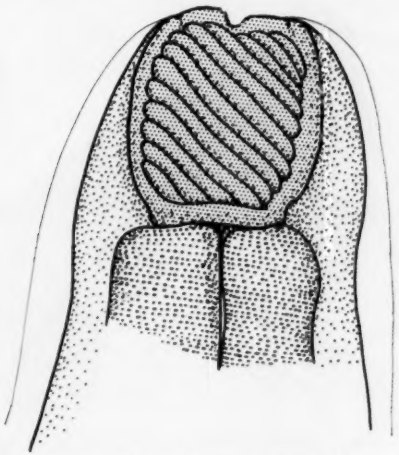

FIGURE 1. Anterior end showing the buccal capsule with its characteristic spiral thickenings, in male and female. ×480

color with conspicuous red-black intestine extending from base of glandular portion of esophagus to anus.

Measurements of our worms (preserved) based upon 25 males and 25 females selected at random are shown below. All measurements are in millimeters.

	Male		Female	
	extremes	average	extremes	average
Body length	10.80–17.65	13.41	13.85–26.50	18.80
Body width	0.125–0.280	0.212	0.235–0.460	0.340
Buccal capsule				
length	0.052–0.079	0.066	0.058–0.083	0.073
width	0.048–0.076	0.061	0.052–0.075	0.066
Esophagus				
muscular	0.280–0.500	0.389	0.350–0.525	0.444
glandular	0.400–0.775	0.610	0.525–0.897	0.685

Male: Posterior end of male (fig. 2) bears symmetrical alar structure which is continuous around slightly curved caudal end. Ala supported by nine pairs of papillae (three pairs preanal and six pairs postanal). All papillae have a flattened terminal crown, from which arises a small thread-like structure that appears to function as means of attachment to ala. Also two pairs of small, modified papillae, that may be called "adanal" papillae, situated at lips of anal aperture (fig. 3). These papillae not mentioned in original description of species by Annereaux. Spicules similar and unequal; right spicule averaging 0.43 mm long and left 0.20 mm long. Tip of tail bears two minute bud-like processes.

Female: Vulva usually situated just posterior to middle of body. In older worms uterus generally packed with eggs and small larvae. Posterior end tapers towards tail and, in region posterior to anus, it constricts and continues as a ventral digitiform process bearing two small terminal projections very similar to those found at posterior end of male (fig. 4).

BEHAVIOR

When the host's entire digestive tract is cut longitudinally and opened, the intestine is usually found to be empty except for its parasites. In such a situation the smallest (immature) worms are normally located at the upper end of the gut, and

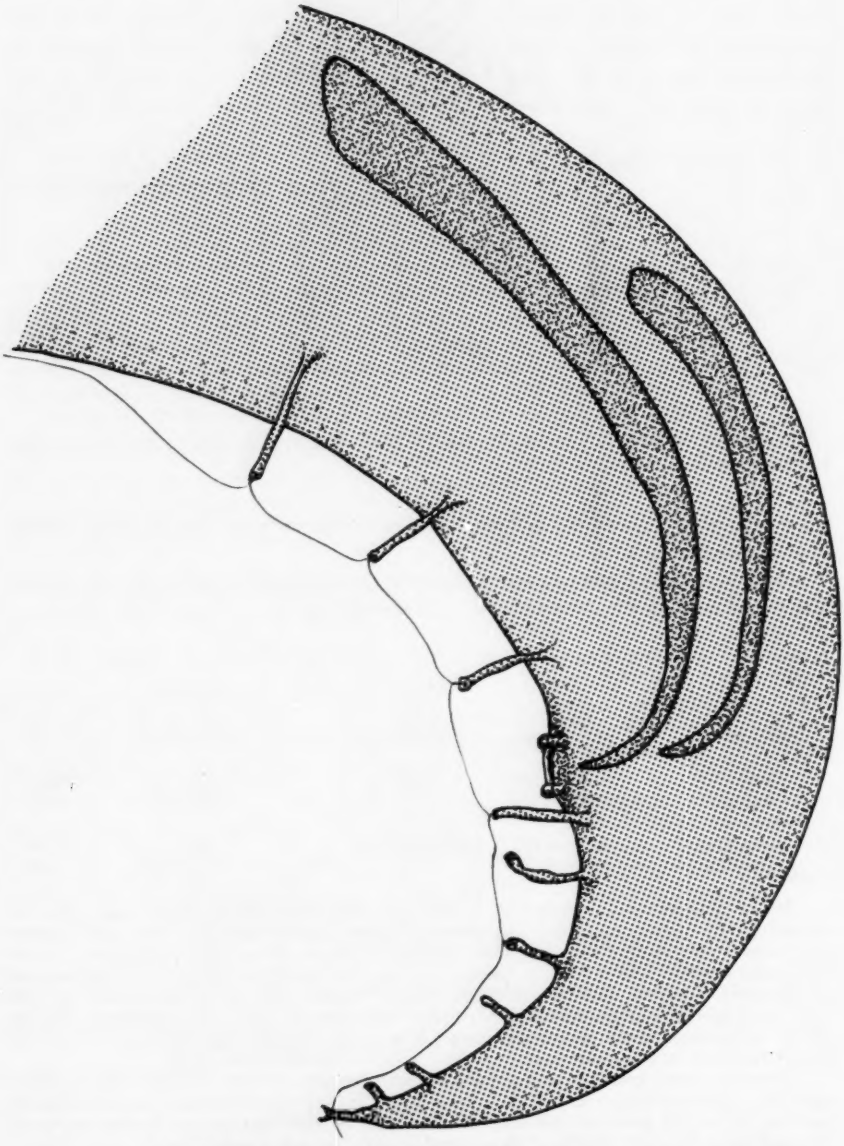

FIGURE 2. Posterior end of the male. Only one side of the transparent alar structure is shown, supported by nine stalk-like papillae. Internal organs are not shown except for the two spicules which point toward the anal opening. ×480

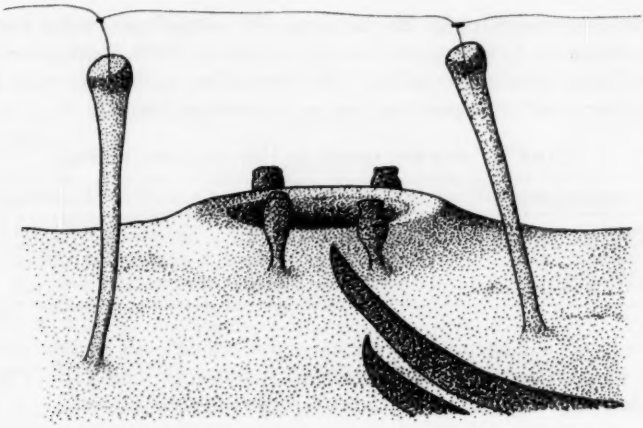

FIGURE 3. Enlarged view of anal opening of male, showing the two pairs of adanal papillae "guarding" the opening. Note the two longer papillae supporting the transparent ala and the tips of the two internal spicules. ×1210

as they become larger they migrate to the lower end. Hence the largest, and most mature, worms are to be found clustered near the anus. All of these nematodes are firmly attached by the buccal capsule to the intestinal mucosa. When a bolus of food is present the larger worms tend to congregate around the food regardless of its position in the gut.

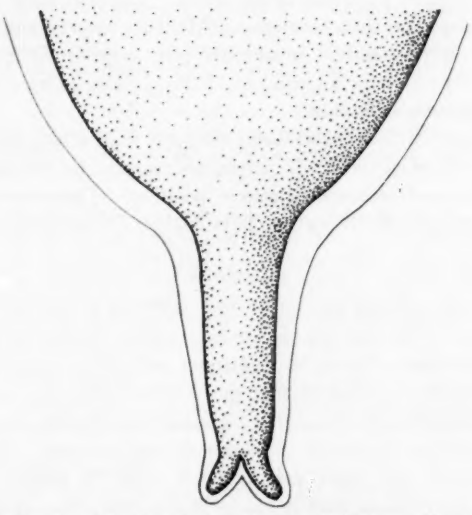

FIGURE 4. Posterior tip of the female showing the digital extension terminating with two small projections. ×1210

A wide geographical range for the genus *Spirocamallanus,* and a tolerance for fish hosts belonging to several families and inhabiting fresh, marine and estuarine environments are indicated in table I. We have added to this record by finding *S. pereidai* in five additional species of fish, as is shown in table II.

TABLE II. *New host records for* Spirocamallanus pereirai.

Species of host	Number autopsied	Number infected
Leptocottus armatus	7	6
Fundulus parvipinnis	21	16
Atherinops affinis	6	3
Girella nigricans	4	4
Gillichthys mirabilis	810	800

The incidence and intensity of infection of *Spirocamallanus pereirai* in *Gillichthys* mirabilis, grouped according to size of host, is shown in table III. The numbers indicate that as the host increases in size it acquires more parasites.

TABLE III. *Incidence and intensity of infection of* Spirocamallanus pereirai *in* Gillichthys mirabilis *grouped according to size of host.*

Size of host in mm.	91–100	101–110	111–120	121–130	131–140	141–150
Number of hosts	16	14	30	33	24	35
Number infected	16	14	30	32	23	35
Average number of worms	3.43	4.42	6.53	8.18	9.82	13.62

Spirocamallanus was present in the gut of *Gillichthys* regardless of the degree of water salinity at the time fish were caught. Experimental evidence of this remarkable tolerance of both fish and parasite to wide environmental changes was obtained by placing fish in glass aquaria with water salinities ranging from fresh water to a salt concentration greater than that of sea water. The fish were left in the aquaria for no less than 3 days and then autopsied. All of the experimental fish were found to be infected by the nematode. In another test, a group of fish was allowed to remain in fresh pond water for a period of 12 days. The entire group was found to have retained the spirocamallanids.

The gut contents of *Spirocamallanus pereirai* was tested for the presence of haemoglobin by subjecting washed, pulverized worms to the benzidine reaction. The results of the reaction were definitely positive. The presence of host blood in the nematode gut may be the cause of its dark red-black color.

SUMMARY

This paper is the first of several dealing with an ecological study of the fish *Gillichthys mirabilis* (the mud sucker or long-jawed Goby) and all of its animal parasites. The fish were collected in an estuary at Goleta, California, and the study is being extended over a period of 3 or more years. Weekly collections during a 2-year period revealed the presence of 15 species of parasites, the most common of which was the intestinal nematode *Spirocamallanus pereirai* (Annereaux) Olsen. Eight hundred out of 810 hosts were infected with the worm. This parasite is world-wide in its distribution, and it has been reported from 14 species of fish. It thus exhibits little host specificity.

As the parasite becomes larger it tends to migrate further posteriad along the gut, and it feeds by sucking host blood with the aid of a buccal capsule equipped with

characteristic spiral thickenings in its wall. No pathogenic effects due to the presence of parasites were apparent. Older hosts generally possessed more worms than did younger hosts. Marked fluctuations in water salinity appeared to have no effect on the host or on its nematode parasites; and seasonal variations of the parasite have not been observed.

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RESEARCH NOTE

RECORDS OF *DRACUNCULUS INSIGNIS* (LEIDY, 1858) FROM RACCOONS IN FLORIDA.

Examination of a raccoon, *Procyon lotor*, trapped by W. L. Jennings in the vicinity of Windsor, Alachua County, Florida, in August 1955 revealed a specimen of *Dracunculus* beneath the skin of the wrist region. The worm was a gravid female with a length of 300 mm and width of 1.2 mm. A second specimen was obtained from a raccoon collected by R. Christensen near Yeehaw Junction, Osceola County, on June 4, 1959. The worm was coiled in the fascia under the skin of the right fore foot just above the pad. It was a gravid female with a length and width of 258 and 1.2 mm, respectively. A random sample of 15 larvae ranged from 504 to 576 microns in total length with a mean of 535 microns.

The two Florida specimens of *Dracunculus* agree closely with descriptions and illustrations of *D. insignis* (Leidy, 1858) given by Chandler (1942, Am. J. Trop. Med. **22:** 153–157), Cheatum and Cook (1948, Cornell Vet. **38:** 421–423), and Hugghins (1958, Proc. S. D. Acad. Sci. **37:** 40–46) and are therefore referred to this species.

North American records of the guinea worm in wild carnivores include the following localities and hosts: Maryland, raccoon (Dikmans, 1948, Proc. Helm. Soc. Wash. **15:** 39–40); Minnesota, mink (*Mustela vison*) and striped skunk (*Mephitis mephitis*) (Erickson, 1946, Am. Midl. Nat. **36:** 494–509; Hugghins, 1958); Nebraska, mink (Chitwood, 1933, J. Am. Med. As. **100:** 802–804); New Hampshire, raccoon (Siegler, 1946, J. Mammal. **27:** 179); New York, raccoon, mink, weasel (*Mustela erminea*), fisher (*Martes pennanti*), otter (*Lutra canadensis*), and striped skunk (Cheatum and Cook, 1948; Chitwood, 1933; Dikmans, 1948; Goble, 1942, J. Mammal. **23:** 221; Hamilton and Cook, 1955, N. Y. Fish and Game J. **2:** 13–35); Pennsylvania (?), raccoon (Leidy, 1858, Proc. Acad. Nat. Sci. Phila. **10:** 110–112); South Dakota, raccoon (Hugghins, 1958); Texas, raccoon (Chandler, 1942); Canada (Ontario), raccoon (Chitwood, 1933). Dikmans (1948) also notes the occurrence of specimens of *Dracunculus* in the U.S.N.M. helminthological collection from muskrats (*Ondatra zibethicus*) from Maryland and North Dakota.

The present specimens apparently constitute the first record of *D. insignis* from Florida and from the southeastern United States in general. The wide separation of the two localities from which infected raccoons were collected suggests that the guinea worm may be generally distributed in the state and may possibly prove to be relatively common when systematic examinations of numbers of raccoons and other carnivores frequenting aquatic habitats are made. —JAMES N. LAYNE, DALE E. BIRKENHOLZ, AND JAMES V. GRIFFO, JR., *Department of Biology and Florida State Museum, University of Florida, Gainesville.*

RESEARCH NOTE

CULTIVATION OF *HISTOMONAS MELEAGRIDIS* IN A MODIFIED TISSUE CULTURE MEDIUM.

This report concerns the cultivation of *Histomonas meleagridis* in a medium that consists essentially of a tissue culture medium, "199," obtained in 10× concentrations from Microbiological Associates, Bethesda, Maryland, and Difco Laboratories, Inc., Detroit, Michigan.

Before use, the "199" was diluted 10-fold with distilled water. The resultant solution was made alkaline by the addition of 1 ml of 5 percent $NaHCO_3$ per 100 ml. It was sterilized by passage through a Seitz filter and then stored in sterile rubber-capped serum bottles under ordinary refrigeration (2 to 10 C) until needed.

For the culturing of the histomonads, this sterilized alkaline solution, in quantities of 4.5 ml, was placed in 15-ml screw-capped culture tubes. To each tube used was added: (a) 0.5 ml of fresh or pasteurized cream sterilized by passage through a Selas filter (maximal porosity radius 0.6 micron); (b) 0.1 ml of an antibiotic mixture containing 100,000 units of penicillin G potassium and 0.1 gm of dihydrostreptomycin sulphate per ml; (c) 0.1 ml of a mycostatin suspension containing 10,000 units per ml; (d) 0.1 ml of a sterile 5 percent solution of $NaHCO_3$; and (e) 1 ml of a nutrient broth culture containing a mixed bacterial flora, originally obtained from turkey cecal droppings. Before inoculation into the culture tubes, the bacterial cultures were subjected for at least 1 hour to the action of 7,100 units of penicillin G potassium and 0.5 gm of dihydrostreptomycin sulphate per ml.

Each culture thus prepared was inoculated with 1 to 2 ml of existing histomonad cultures, and then overlaid with 0.5 ml of liquefied white petroleum jelly from a flamed hypodermic syringe. The tubes were then capped and incubated at a temperature of 38 to 40 C. Subcultures were made, as described above, every 48 to 72 hours.

Microscopical examinations to determine the status of the parasites were made by removing a small amount of the medium from each culture tube with a sterile Pasteur pipette. The number of histomonads observed in any one examination of 300 cultures selected at random ranged from 30 per 1 microscopic field (15× by 20×) to less than 1 per 10 fields, with an average of 2 to 3 organisms per field. Approximately one-half of the cultures had at least one organism per field. The protozoa failed to develop in less than 3 percent of all inoculated cultures.

Tests for sterility just before subculturing were made by transferring 0.5 to 1.0 ml of the medium from each histomonad culture tube to thioglycollate broth, dextrose nutrient broth containing a pH indicator and fermentation tube, and, occasionally, to mycophil broth (Baltimore Biological Laboratory, Inc., Baltimore, Maryland). The thioglycollate and the mycophil broths were observed for 2 to 7 days, and the dextrose broth for 7 days. As a further test, approximately 1.0 ml from each of those thioglycollate broths that appeared to be negative for bacteria was inoculated into fresh thioglycollate broth tubes and maintained for another 2 to 4 days. These sterility tests have indicated probable bacterial growth in less than 5 percent of the histomonad cultures thus far made.

However, histomonads have now been maintained through more than 40 consecutive subcultures without evidence of bacterial growth. It is, therefore, presumed that the histomonads involved are capable of surviving and reproducing in the modified "199" medium without the presence of demonstrable live bacteria, as indicated by the sterility tests that were employed.

In similar experiments, it was found that histomonads failed to multiply if the bacterial nutrient broth cultures were autoclaved at 15 pounds pressure for 20 minutes prior to being introduced into the modified "199" medium. Seitz-filtrates of the bacterial nutrient broth cultures also failed to support histomonad growth. In the light of these findings, it appears that a heat-labile intracellular factor(s) present in turkey cecal bacteria is required for the successful growth and reproduction of *H. meleagridis*.—ELLIOTT LESSER, *Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.*

SPRING INCIDENCE OF RUFFED GROUSE BLOOD PARASITES*

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The summer and fall occurrence of blood parasites in ruffed grouse (*Bonasa umbellus*) of the north-central states has been well documented (Fallis, 1945; Erickson et al, 1949). However, no comprehensive spring surveys of the incidence of these parasites have been reported. This paper presents information derived from the examination of blood films taken from cocks trapped in the spring of 1954 and 1955, comparative data from smears made from fall-shot birds, and pertinent data from an experiment on leucocytozoonosis relapse conducted by Kenneth Flakes** on winter-trapped ruffed grouse.

METHODS

Individual breeding cocks caught once during the months of April and May in Rusk, Sawyer and Dunn Counties, Wisconsin, were bled for blood smears, banded, and then released as a part of a study on natural mortality rates. For trapping techniques see Dorney and Mattison (1956). All blood samples were taken from the brachial vein; the time was usually between 8 and 10 a.m. The number of smears examined totaled 64 in 1954 and 100 in 1955. By the use of several anatomical measurements (Dorney and Holzer, 1957) cocks were classified as either juvenile (10 to 12 months old) or adult (2 years or more) in the 1955 spring sample.

Each spring blood smear, stained with Wright's stain, was examined for 20 to 30 minutes under magnifications of 100, 430 and 970 (oil). During a portion of the allotted time, approximately 4,000 red blood cells on each slide were counted (oil immersion), and the percentage of cells infected with *Haemoproteus*, if any, recorded. Only gametocytes of *Haemoproteus* mature enough to contain pigment granules were tallied to avoid any possible confusion with young trophozoites of *Plasmodium*.

The severity of infection with *Leucocytozoon* was recorded on a subjective scale of 1 to 4, with infections of a 4 magnitude equivalent to about one gametocyte for each two oil immersion fields (970×). *Trypanosoma* or microfilaria infections were recorded without regard to the degree of parasitemia.

Late summer-fall blood smears were taken from 373 birds shot during the period 1948 to 52 and are reported herein for comparative purposes. They were examined by the following people: Banner Bill Morgan, 1949; Kenneth Flakas, 1948, 1950–51; Geoffrey Lord, 1952. Wright's stain was used, and about 10 to 15 minutes allotted for examination of each smear under low, high dry, and oil immersion magnifications.

Sixteen ruffed grouse trapped in Dunn County, Wisconsin, in 1952 during the month of February were shipped express 150 miles to the state laboratory at Poynette, Wisconsin. The first seven birds received on February 5, 1952, were taken into the laboratory and caged together for 37 days at room temperature. The remainder received on February 13 and 27 were divided into two groups, with three birds added to those caged inside, and the other six caged at outside temperatures (30 to 38 F) for 15 days. Flakas examined blood smears from these birds at weekly intervals to study the appearance of gametocytes of *Leucocytozoon* and *Haemoproteus* in peripheral blood.

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** Progress reports and personal notes of Flakas, former Wisconsin Conservation Department pathologist.

RESULTS

The spring occurrence of the five blood parasites found in this study is indicated (table I) by bi-monthly periods. Data from both 1954 and 1955 are combined

TABLE I. *Seasonal incidence of blood parasites, 1954 and 1955.*

Time period	No. of birds	Percent infected with				
		Haemoproteus	*Leucocytozoon*	*Trypanosoma*	*Microfilaria*	*Plasmodium*
April 1 to 15	21	48	71	33	38	–
April 16 to 30	72	57	82	31	25	–
May 1 to 15	34	44	91	41	29	3
May 16 to June 4	37	81	97	27	46	–
Total and weighted average	164	58	86	32	32	1

because of no major differences. The percentage of positive blood smears for *Haemoproteus* and *Leucocytozoon* increased from early April to late May in contrast to the other three parasites. In addition to this increase in blood smears positive for *Haemoproteus* and *Leucocytozoon* in May, the numbers of circulating gametocytes for these protozoa in positive birds also increased at this time (table II).

TABLE II. *Mean spring level of* Haemoproteus *and* Leucocytozoon *parasitemias in infected birds, 1954 and 1955.*

Time period	*Haemoproteus*	*Leucocytozoon*
April 1 to 15	2*	1.3**
April 16 to 30	2	1.5
May 1 to 15	21	1.8
May 16 to June 4	150	1.8

* Number of infected RBC per 10,000.
** Relative level of abundance, see text.

When the 1955 spring sample was segregated by age groups into juvenile and adult cocks (table III), no difference was apparent for *Haemoproteus, Plasmodium,* and microfilaria. However, the occurrence of *Leucocytozoon* and *Trypanosoma* was lower in juveniles (birds hatched previous summer) than in adults ($P < 0.01$). Since the ratio of juveniles and adults in table III was approximately equal in each month's sample, the difference in the percentage of positive blood smears is presumed to represent a small but constant difference between age groups.

A comparison between the occurrence of blood parasites in these spring samples and reported fall studies (table IV) shows that *Haemoproteus* is an uncommon summer-fall parasite (8 percent) in sharp distinction to its occurrence in over half the cocks in spring. *Leucocytozoon,* although the most common parasite seen in peripheral blood smears in the summer-fall period (60 percent), also is found in an even greater proportion of cocks in spring (86 percent). Similarly, trypanosomes and microfilaria, almost absent in fall smears, are commonly seen in spring. *Plas-*

TABLE III. *Incidence of blood parasites in juvenile and adult cock ruffed grouse, spring 1955.*

Age	Sample size	Percent infected with				
		Haemoproteus	*Leucocytozoon*	*Trypanosoma*	*Microfilaria*	*Plasmodium*
Juvenile	30	47	70*	13*	20	3
Adult	70	53	96*	50*	34	0

* Age groups differ significantly at 1 percent level.

TABLE IV. *Comparative incidence of blood parasites in fall and spring.*

Area	Sample size	Percent infected with				
		Haemoproteus	*Leucocytozoon*	*Trypanosoma*	*Microfilaria*	*Plasmodium*
		Summer-fall				
Wisconsin*	373	8	47	2	1	3
Minnesota**	163	0	70	1	4	0
Ontario***	106	16	63	6	7	7
Total and Average	642	8†	60†	3†	4†	3
		Spring				
Wisconsin*	164	58†	86†	32†	32†	1

* This study.
** Erickson, et al 1949.
*** Fallis, 1945.
† Time periods differ significantly at the 1 percent level.

modium, by way of contrast, is uncommon at all seasons sampled. This fall and spring difference cannot be explained by changes in the proportion of juveniles and adults sampled, since a high percentage of both age groups in spring are heavily parasitized (table III). Although the spring and fall samples (table IV) are drawn from different year periods, the stability exhibited between the 12 years represented in these fall samples (Erickson, 1953, and this study) makes it appear unlikely that these high percentages in spring are chance variants from the mean that happened to occur during these 2 years (1954 and 1955).

The experiment conducted by Flakas on leucocytozoonosis relapse in 14 wild-trapped ruffed grouse in February and March 1952 is summarized in table V. No

TABLE V. *Ratio of wild ruffed grouse showing* Leucocytozoon *and* Haemoproteus *in peripheral blood at intervals following caging in 1952.*

Date	*Leucocytozoon*	*Haemoproteus*
February 5	0/7	0/7
February 13	0/7* **	0/7
February 21	2/5*	0/5
February 27	7/12**	0/12
March 4	7/11*	0/11
March 10	7/10*	1/10
March 14	5/7*	1/7

* Deaths reduced number of birds.
** New wild-trapped birds added.

difference in ratios of birds positive for *Leucocytozoon* was observed between the seven birds held in the heated laboratory and the seven held outside; therefore, both groups were combined. Numbers of birds fluctuated due to deaths of a traumatic nature and the addition of newly trapped wild replacements. According to these limited samples, *Leucocytozoon* was absent early in February, but was found in the majority of birds in March, documenting the occurrence of a late-winter relapse for *Leucocytozoon* since black fly vectors (*Simulium*) would be absent in the laboratory.

The appearance of *Haemoproteus* gametocytes in one bird in March (table V) also suggests relapse may have occurred with this parasite. However, since the vector(s) responsible for transmission of *Haemoproteus* in ruffed grouse are un-

known, transmission from ectoparasites present on the caged bird remains as an alternative possibility.

To understand the epizootiology of spring blood parasites, three calculations were made: (1) A cock activity curve of territorial drumming computed by timing the number of these territorial drumming sounds heard at 15 locations throughout the spring months of 1954 and 1955. (2) A chick hatching curve compiled from data of Hale and Wendt (1951) and personal field observation. (3) An index of abundance of *Haemoproteus* and *Leucocytozoon* calculated by multiplying percent occurrence (table I) times their respective parasitemias (table II) for each time period. These computed breeding phenologies and parasite indices (fig. 1) present a graphic portrayal of the level of infection in the breeding cock population in relation to reproductive events in the spring.

The epizootiological sequence is as follows: *Haemoproteus* remains low in April, (fig. 1), then surges upward as drumming activity peaks, and finally subsides in late

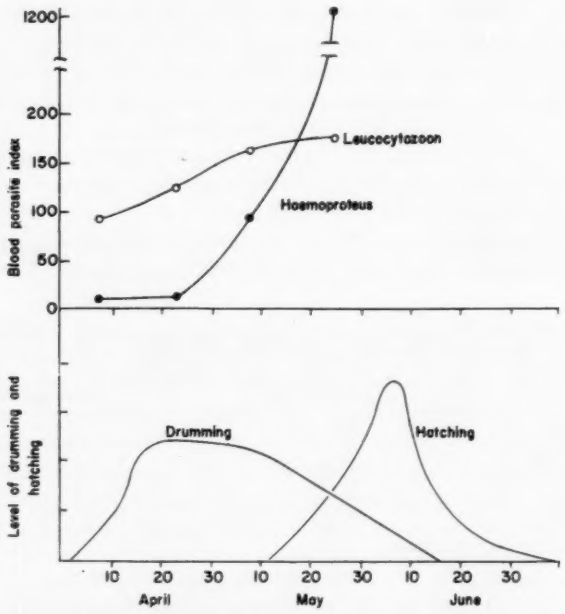

FIGURE 1. Chronological index of abundance of *Haemoproteus* and *Leucocytozoon* in breeding cocks, in relation to cock-drumming activity and hatching of chicks.

May. During this same time period, the *Leucocytozoon* index approximately doubles. Hatching of young birds gets underway in the last half of May and peaks in early June, coming just after these known high parasitemias of *Leucocytozoon* and *Haemoproteus*.

In regard to vectors, Fallis and Bennett (1958) have reported *Simulium latipes* and *S. aureum* as appropriate vectors for transmitting *Leucocytozoon* to ruffed grouse. *S. latipes* adults typically appear in northern Wisconsin in late May or early June, and *S. aureum* in early June (John Anderson, pers. comm.), coinciding with

high adult male parasitemias and the hatching of young grouse. This time sequence would appear to provide ideal conditions for the efficient transference of *Leucocytozoon* to susceptible young. The same might be true for *Haemoproteus,* presuming the appropriate vectors are present at this time. If the adult hen is similarly infected in spring, this would of course increase the probability of vectors securing gametocytes for eventual transmission to the young.

DISCUSSION

Haemoproteus

The profound increase in incidence and numbers of circulating gametocytes of *Haemoproteus* from April to June (tables I, II and fig. 1) is a phenomenon that has not been observed previously in ruffed grouse. Whether these high parasitemias represent true relapse, reinfection, or both cannot be answered with certainty since vector responsible for transmission is unknown. The conclusion that relapse is strongly involved is supported by the appearance of parasitemias in early April (table I) before insect transmission seems likely, and the parasitemia in one caged grouse in March.

Other workers have reported the chronic nature of this protozoan as well as increased numbers of gametocytes in peripheral blood in spring. Herman and Bischoff (1949), working with caged California quail (*Lophortyx californica*), demonstrated that quail could remain *Haemoproteus* carriers up to 4 years with intermittent periods of relapse. Borg (1953) noted an increase in proportion of positive smears of *Haemoproteus* for Swedish grouse in spring and summer. Huff (1942) reported positive *Haemoproteus* blood smears from 4 adult mallards on April 1 that had been negative on March 6. Kestrels (*Falco tinnunculus*) in Europe also exhibited increased parasitemias in spring with a decrease in fall and winter (Wasielewski and Wulker, 1918).

Coatney (1932) felt that relapse of *Haemoproteus columbae* in the pigeon was initiated when host resistance was reduced, although he was unsuccessful in producing it with artificial stressing agents. Evidence from this study supporting Coatney's hypothesis might be mentioned. May temperatures in 1954 during the peak of the breeding season were extremely cold, in contrast to a warm May in 1955. The percentage of red blood cells containing *Haemoproteus* gametocytes was 2.4 times higher in late May 1954 as compared to levels at the same time in 1955. Perhaps the abnormally cold temperatures directly and/or indirectly may have acted as an additional stress causing a more severe "relapse" in 1954.

In regard to vectors, Fallis (pers. comm.) feels that hippoboscid flies are not common enough in spring to explain transmission of *Haemoproteus* to young passeriform birds in Ontario. In handling ruffed grouse in spring, hippoboscids were rarely seen; they were more common in fall. Recently, *Culicoides* have been shown to transmit *Haemoproteus* in waterfowl (Fallis and Wood, 1957). An insect abundant in late May and June such as *Culicoides* (Fallis, pers. comm.) coinciding with high parasitemias in grouse would be a more likely vector than hippoboscids.

Leucocytozoon

The *Leucocytozoon* relapse in ruffed grouse apparently begins in March (table V) and by April is well advanced (tables I, II). The further increase in incidence in May (table I) could represent both relapse and new infections since Fallis (pers.

comm.) has observed black flies feeding on adult ruffed grouse in early May. Infection in young chicks by the end of June noted in Ontario (Fallis and Bennett, 1958) suggests a similar spring relapse must occur in breeding males and/or females in Ontario.

In both waterfowl and sharp-tailed grouse (*Pedioecetes phasianellus*) the numbers of circulating gametocytes of *Leucocytozoon* increase during the spring (Huff, 1942; Chernin, 1952; Cowan and Peterle, 1957). Borg (1953) found in peripheral blood of Swedish grouse a similar fluctuation in *Leucocytozoon*, with a higher percent of infected birds in May and June and a lower rate in January–February. Based on data from figure 1 and tables IV and V, ruffed grouse follow this same pattern in Wisconsin.

Trypanosoma

Two morphologically distinct trypanosomes were seen, one with and one without myonemes. Both measured between 48 and 60 microns (average 54). The form with myonemes often had a heavier undulating membrane and a more prominent nucleus. Whether these trypanosomes are distinct species or represent stages in maturation is not known. It was not uncommon to see both types in the same blood smear. Coatney and Roudabush (1937) reported two different-sized trypanosomes in passeriform birds, the larger form with myonemes. These authors found transitional forms and felt they all belonged to the same species (*T. avium*).

Only one trypanosome was seen in many of the blood smears examined. This suggested that if repeated blood smears or inoculation of whole blood into culture media could be made, the 32 percent incidence observed in spring would be much higher. Diamond and Herman (1954), by culturing bone marrow of 174 Canada geese (*Branta canadensis*), were able to diagnose trypanosomiasis in about 26 percent of these birds; all blood smears were negative. The low incidence of trypanosomes in our fall samples (3 percent) raises the question as to the possibility that spring breeding activity may also result in relapse, similar to that noted for *Leucocytozoon*, thereby enhancing the likelihood of transmission to young chicks.

Pathogenicity

Much speculation has been raised as to the possible death loss in ruffed grouse which may be attributed to the various blood protozoa. Population studies (Dorney, 1959) made in conjunction with the blood studies in northern Wisconsin help in part to answer this question. The following general pattern of reproduction and survival occurred over an 8-year period (1950–1958).

Average complete clutch size (38 observations) was 10.5. Mortality of young measured by monthly brood observations indicated an average 18 percent summer loss (June to August). Fall sex ratios of adults averaged 54 males: 46 females. This variance from a 50: 50 ratio was presumed due to a heavier spring-summer mortality of breeding hens, since sex ratios of young are 50: 50.

The specific mortality and production during the 2 years 1954 and 1955 were as follows. In 1954, the spring breeding population was at a low point in the 10-year period, 1949–1958. Weather was extremely cold in May with a 6-inch snowfall interrupting cock drumming early in the month. Production of young was poor (average summer brood size 6.8) and adult sex ratios in fall considerably distorted, 58 males: 42 females, suggesting a heavy summer hen loss. Calculated adult male

survival to the next spring (1955) was, by way of contrast, above average, with a 24 percent mortality in this 12-month period (average annual adult male mortality, 56 percent).

In 1955 the spring was strikingly different than that of 1954, with above average temperatures. Production of young was high with summer brood sizes averaging 8.5. Adult female loss in summer was low as indicated by a fall sex ratio of 52 males: 48 females. Yet, adult male survival over the 12-month period to spring 1956 was considerably poorer with a calculated mortality of 48 percent, double that of the preceding year.

As already mentioned, the numbers of circulating gametocytes of *Haemoproteus* in the cocks in 1954 were 2.4 times higher than in 1955 and for *Leucocytozoon* 20 percent higher. However, the minimal cock mortality (24 percent) from 1954 to 1955 provides direct evidence that a universal infection and high parasitemia of *Haemoproteus* and *Leucocytozoon* in 1954 were not fatal to any significant proportion of these cocks (tables I, II).

The increased adult female mortality in 1954 could conceivably be due to the combined effect of unfavorable weather in May for nesting, and infection with blood protozoa. However, the lack of blood smears from adult hens relegates this to the role of speculation. If, however, blood protozoa cause mortality, the breeding female may be more vulnerable than the male owing to concurrent reproductive stresses of egg laying and incubation. Kabat, Meyer, Flakas and Hine (1956) have experimentally demonstrated that hen pheasants during and just after laying and incubation have minimal adaptation energy when the non-specific stress of starvation is applied. Protozoan diseases in grouse could act as a non-specific stressing agent similar to experimental starvation in pheasants.

The general high survival of young throughout the summer months over an 8-year period eliminates the possibility that the blood protozoa are causing large-scale mortality to chicks in most years. However, the reason for above-average mortality in 1954 to chicks remains unknown. The general low summer mortality of young birds is not due to lack of infection, since *Leucocytozoon* and *Haemoproteus* are abundant in juveniles (tables III and IV).

In conclusion, all currently available field observations on adults and juveniles suggest these blood protozoa are not involved in causing heavy mortality to ruffed grouse. Both Borg (1953) in Sweden and Erickson (1953) in Minnesota have similarly concluded that *Leucocytozoon* is not an important cause of death in wild grouse. Their conclusions were based on the necropsy of birds found dead in the field and the stability of the occurrence of *Leucocytozoon* in both increasing and decreasing populations.

In view of the known mortality caused by some of these same protozoa in both domestic avian and mammalian hosts, it seems likely that considerable host energy must be expended by ruffed grouse in achieving a host-parasite balance. The net effect of this chronic disease condition on longevity, observed summer weight loss in hens, or wariness may well prove important to the population. Population density further complicates the study of these host-parasite relationships, since fall-winter mortality increases and production of young birds decreases when ruffed grouse numbers are high (Bump et al, 1947; Dorney, 1959). Thus factors unimportant in years of low population density may become limiting at high densities. If ruffed grouse hatched in isolation can be stocked successfully on islands at present

without grouse, as is currently contemplated in Michigan, detailed ecological study of these presumably disease-free birds should prove enlightening.

SUMMARY

Observed infections in blood smears collected from ruffed grouse in fall, winter, and spring were interpreted to indicate increased incidence of *Haemoproteus, Leucocytozoon, Trypanosoma,* and microfilaria in spring. *Leucocytozoon* and *Haemoproteus* were essentially absent in blood smears made in February, but were found in increasing numbers from March to June. This increased occurrence of blood protozoa and microfilaria in the breeding season provides an ideal epozootiological link between infection in adults and newly hatched chicks. Concurrent field information on production and survival of ruffed grouse indicated little evidence of direct large-scale mortality due to these blood protozoa.

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SOME PARASITES OF CATFISHES FROM KANSAS*

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The study here reported on was undertaken to gain specific knowledge of parasites of the channel catfish, *Ictalurus punctatus* (Rafinesque), black bullhead, *I. melas* (Rafinesque), and yellow bullhead, *I. natalis* (LeSueur), in rivers, creeks lakes, and ponds of northeastern Kansas.

The members of the genus *Ictalurus* were chosen for this study because they are widely distributed in the state and this extensive distribution permits a study of parasitism in various ecological situations in each season of the year. In addition, the channel catfish is sought by fishermen as a game-fish and a knowledge of its parasites could prove valuable to persons engaged in fisheries research.

MATERIALS AND METHODS

One hundred thirty-five catfishes were collected and autopsied between March 29, 1956, and February 14, 1957. Fish were collected in eight counties of Kansas (Jackson, Shawnee, Wabaunsee, Douglas, Franklin, Miami, Linn, and Chase) mainly by using seines, traps, or gill nets, the type of habitat determining the best procedure. Other methods employed were angling, dip-netting, poisoning with rotenone, and shocking by an electric current from a portable generator.

In all but five instances the fish were retained alive for autopsy; this was found to be the only satisfactory method for studying internal parasites. If fish are frozen or preserved in formalin the parasites die in a contracted state, and those in the gut are affected severely by the digestive enzymes. Hosts were examined first for flesh-parasites and then eviscerated; each organ was placed in a separate container of 0.7 percent solution of sodium bicarbonate. Under a binocular dissecting microscope the tissues were dissected and the parasites freed.

The fixatives employed were AFA Solution (formalin, ethanol, and acetic acid), 70 percent ethanol, Bouin's Fluid, or 10 percent formalin. Flatworms, acanthocephalans, and leeches were stained in alum cochineal, aceto-carmine, or hematoxylin, and mounted in balsam. Best results were obtained with nematodes and copepods by clearing in glycerine and mounting unstained in glycerine-jelly.

ANNOTATED LIST OF PARASITES

TREMATODA

Cleidodiscus floridanus Mueller, 1936 (Monogenea: Dactylogyridae)

Developmental stage: Adult.
Hosts: All three species of *Ictalurus.*
Organ parasitized: Filaments of the gills.
Incidence: 41.5 percent (50 percent in channel catfish; 71.5 percent in yellow bullheads; 33 percent in black bullheads).
Intensity: 1 to 366 per host; average 18.5 per infested host.

Phyllodistomum caudatum Steelman, 1938 (Digenea: Gorgoderidae)

Developmental stage: Adult.
Hosts: Yellow bullhead, black bullhead.
Organ parasitized: Urinary bladder.
Incidence in infected species: 10.9 percent (14.3 percent in yellow bullheads; 10.5 percent in black bullheads).
Intensity: 1 to 7 per host.

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Phyllodistomum lacustri (Loewen, 1929)

Developmental stage: Adult.
Hosts: Channel catfish.
Organ parasitized: Urinary bladder.
Incidence in infected species: 7.7 percent. (All infected fish were from Prairie Lake, Jackson Co.)
Intensity: 1 to 7 per host.

Phyllodistomum carolini Holl, 1929

Developmental stage: Adult.
Host: Black bullhead.
Organ parasitized: Urinary bladder.
Incidence in infected species: 6.6 percent.
Intensity: 1 to 14 per host.

Crepidostomum ictaluri (Surber, 1928) (Digenea: Allocreadiidae)

Developmental stage: Adult.
Hosts: Channel catfish, black bullhead.
Organ parasitized: Anterior two-thirds of the intestine.
Incidence in infected species: 27.3 percent (42.4 percent in channel catfish; 17 percent in black bullheads).
Intensity: 1 to 235 per host; average 65.3 per infected host.

Plagioporus sp. (Digenea: Allocreadiidae)

Developmental stage: Adult.
Host: Black bullhead.
Organ parasitized: Intestine.
Incidence: A single immature specimen was collected.

Alloglossidium corti (Lamont, 1921) (Digenea: Allocreadiidae)

Developmental stage: Adult.
Hosts: Yellow bullhead, black bullhead.
Organ parasitized: Intestine (74 percent in posterior one-third).
Incidence in infected species: 4.8 percent.
Intensity: 1 to 13 per host.

Pisciamphistoma stunkardi (Holl, 1929) (Digenea: Paramphistomidae)

Developmental stage: Adult.
Host: Yellow bullhead.
Organ parasitized: Intestine.
Incidence: 4 specimens collected from a single host.

Clinostomum marginatum (Rudolphi, 1819) (Digenea: Clinostomatidae)

Developmental stage: Metacercaria.
Hosts: Channel catfish, black bullhead.
Tissue parasitized: Skin and body muscles, especially at the bases of fins.
Incidence in infected species: 5.2 percent.
Intensity: 1 to approximately 150 per host.

CESTODA

Proteocephalus ambloplitis (Leidy 1887) (Proteocephala: Proteocephalidae)

Developmental stage: Plerocercoid.
Hosts: All three species of *Ictalurus.*
Organs and tissues parasitized: Liver, spleen, mesentery, abdominal fat, connective tissue of the eye.
Incidence: 26 percent (29 percent in channel catfish; 14.3 percent in yellow bullheads; 26.3 percent in black bullheads).
Intensity: 1 to 29 per host.

Corallobothrium fimbriatum Essex, 1927 (Proteocephala: Proteocephalidae)

Developmental stages: Plerocercoid and adult.
Hosts: Channel catfish, black bullhead.
Organ parasitized: Intestine.

Incidence in infected species: 16.5 percent (17.3 percent in channel catfish; 15.8 percent in black bullhead).
Intensity: 1 to 21 per host.

Corallobothrium giganteum Essex, 1927

Developmental stages: Plerocercoid and adult.
Hosts: All three species of *Ictalurus.*
Organ parasitized: Intestine.
Incidence: 20.7 percent (29.4 percent in channel catfish; 15.8 percent in black bullheads; 14.6 percent in yellow bullheads).
Intensity: 1 to 61 per host.

ACANTHOCEPHALA

Neoechinorhynchus sp. (Eoacanthocephala: Neoechinorhynchidae)

Developmental stage: Adult.
Host: Yellow bullhead.
Organ parasitized: Intestine (completely encysted in wall).
Incidence: 2 specimens were collected from a single host.

Pomphorhynchus bulbocolli Linkins, 1919 (Paleacanthocephala: Echinorhynchidae)

Developmental stage: Juvenile.
Host: Yellow bullhead.
Tissue parasitized: Mesentery.
Incidence: 150 specimens collected from a single host.

NEMATODA

Spinitectus gracilis Ward and Magath, 1917 (Spiruroidea: Spiruridae)

Developmental stage: Adult.
Hosts: All three species of *Ictalurus.*
Organs parasitized: 87 percent in the stomach; 13 percent in the intestine.
Incidence: 14.8 percent (13.5 percent in channel catfish; 14.3 percent in yellow bullheads; 15.8 percent in black bullheads).
Intensity: 1 to 56 per host.

Camallanus oxycephalus Ward and Magath 1917 (Spiruroidea: Camallanidae)

Developmental stage: Adult.
Host: Channel catfish.
Organ parasitized: Intestine.
Incidence: 6 specimens were collected from a single host.

Dichelyne robusta (Van Cleave and Mueller, 1932) (Spiruroidea: Cucullanidae)

Developmental stage: Adult.
Host: Channel catfish.
Organ parasitized: Intestine.
Incidence: 4 specimens were collected from a single host.

Contracaecum sp. (Ascaroidea: Heterochelidae)

Developmental stage: Larva.
Host: Yellow bullhead.
Tissue parasitized: Mesentery.
Incidence: 3 specimens were collected from a single host.

Unidentified type 1 larval nematode

Brief description: Larvae measuring 3.8 mm in length by 0.1 mm in diameter and with a simple pharynx measuring 0.88 mm in length enclosed individually in yellow colored cysts.
Hosts: Black bullhead, yellow bullhead.
Tissue parasitized: Mesentery.
Incidence in infected species: 7 percent.
Intensity: 1 to 3 per host.

Unidentified type 2 larval nematode

Brief description: Larvae measuring 1 mm in length by 0.33 mm in diameter and having no

distinguishable pharynx enclosed, in groups of approximately 100, in cream colored cysts measuring 0.8 mm in diameter.
Host: Black bullhead.
Tissue parasitized: Mesentery near bile duct.
Incidence: Approximately 200 specimens were collected from a single host.

HIRUDINEA

Piscicolaria reducta Meyer 1940 (Rhynchobdellida: Piscicolidae)

Developmental stage: Adult.
Hosts: Channel catfish, black bullhead.
Tissues parasitized: Skin surface at bases of fins and ventral side of head.
Incidence in infested species: 3 percent.
Intensity: 1 to 3 per host.

Placobdella montifera Moore, 1917 (Rhynchobdellida: Glossiphoniidae)

Developmental stage: Adult.
Host: Black bullhead.
Tissue parasitized: Skin surface.
Incidence: 1 specimen collected.

COPEPODA

Achtheres pimelodi Krøyer, 1863 (Eucopepoda: Lernaeopodidae)

Developmental stage: Adult.
Host: Black bullhead.
Organ parasitized: Filaments of the gills.
Incidence in infested species: 15.8 percent.
Intensity: 1 to 6 per host.

Ergasilus versicolor Wilson, 1911 (Eucopepoda: Ergasilidae)

Developmental stage: Adult.
Hosts: All three species of *Ictalurus*.
Organ parasitized: filaments of the gills.
Incidence: 30.4 percent (30.8 percent in channel catfish; 14.6 percent in yellow bullheads; 31.6 percent in black bullheads).
Intensity: 1 to 482 per host.

ECOLOGY OF THE PARASITES

One hundred twenty-four (91.9 percent) of the catfishes were infected with at least one species of parasite. Table I indicates the extent of parasitism for each species of *Ictalurus*.

Essex and Hunter (1926) examined 127 catfish of five species (including black bullheads and channel catfish) and found 54 (42.5 percent) parasitized. Bangham and Venard (1942) report parasites in 97 percent of the catfishes of Reelfoot Lake, Tennessee; all of the black bullheads and channel catfish were infected. Wilson (1953) found 54.5 percent of the channel catfish and yellow bullheads parasitized in Leavenworth County Lake, Kansas.

Of the 7,127 parasites collected in the present study, 89.3 percent inhabited parts of the body of the host where the eggs or larvae of the parasite could be easily passed into the water. The remaining 10.7 percent were found in closed systems of the body and the fish would have had to be eaten by another animal in order for the life cycle of the parasite to be completed.

Ponds, lakes, and streams are different in their physical characteristics and differ somewhat in parasitic faunas. Table I compares parasitism in the four types of habitat for each species of *Ictalurus*, and infection by external and internal parasites.

TABLE I. *Parasitism in various habitats.*

Species of catfish and habitats	Number examined	Number parasitized	Percent parasitized	Number with internal parasites only	Number with external parasites only	Number with internal and external parasites
Ictalurus punctatus	52	48	92.4	4	7	37
Lakes	12	12	100	0	0	12
Ponds	3	2	66.7	1	0	1
Rivers	35	32	91.5	4	7	21
Creeks	2	2	100	2	0	0
Ictalurus melas	76	69	90.8	29	6	34
Lakes	0	—	—	—	—	—
Ponds	26	24	92.3	20	0	4
Rivers	7	7	100	2	2	3
Creeks	43	38	88.3	7	4	27
Ictalurus natalis	7	7	100	2	2	3
Lakes	0	—	—	—	—	—
Pounds	1	1	100	1	0	0
Rivers	3	3	100	1	0	2
Creeks	3	3	100	0	2	1
Total	135	124	91.9	35	15	74
Lakes	12	12	100	0	0	12
Ponds	30	27	90	22	0	5
Rivers	45	42	93.3	7	9	26
Creeks	48	43	89.5	9	6	28

The farm-ponds studied were impoundments that had no continuous connections with creeks or rivers; thus each pond was a relatively independent community in terms of fish parasites. External parasites (leeches, copepods, and monogenetic trematodes) were less common on fishes from ponds than on those from rivers and creeks. *Cleidodiscus floridanus* was found on only five catfish from ponds, as compared with 51 catfish from rivers and creeks. *Placobdella montifera, Piscicolaria reducta,* and *Achtheres pimelodi* were absent from pond-fish, and *Ergasilus versicolor* was found on only one channel catfish from a pond. Ponds probably are ecologically fit for these parasites, but because ponds are independent of rivers and because there is no intermediate host necessary that could transport larval stages to the ponds from rivers, the parasites have failed to be introduced.

The creeks and rivers studied varied from 4 feet to more than 200 feet in width. The only parasites found to be unique to creeks and rivers were leeches and these were present on fish taken from water where there was only a slight current.

The two lakes from which catfish were obtained had surface areas of 78 and 178 acres. One lake was an impoundment entirely independent of creeks and rivers, whereas the other had frequent connection with a stream. *Phyllodistomum lacustri* was the only parasite found to be limited to lake fish and the highest infestations of *Ergasilus versicolor* were on fishes from lakes.

The seasonal distribution of parasites is significant. The peak of parasitism, as determined by percentage of fish parasitized and number of parasites per fish, occurred in the months of June, July, and August. In August, although 100 percent of the fish examined were parasitized, there was an average of only 27 non-encysted parasites per fish as compared with 79 and 87 per fish in June and July respectively. The lowest degree of parasitism was in March and April. These observations indicate that parasites are shed by the host during that time of the year when food consumption is presumably the lowest. Conversely, parasitism is highest during the months when food consumption is the highest; thus, more ova and larval stages of parasites are ingested.

There is no overall correlation of the size of the fish and the extent of parasitism. The degree to which individual hosts were parasitized was influenced more by season and habitat than by size of the fish. In a series of catfish collected in the same season from the same locality, the larger fish usually had more parasites than the smaller ones; however, this difference was minor and wholly obscured by seasonal and environmental differences.

The only situation where extensive parasitism may have caused death involved a yellow bullhead brought in dead from a pond. Upon autopsy 150 juvenile acanthocephalans (*Pomphorhynchus bulbocolli*) and four larval nematodes (*Contracaecum* sp.) were found encysted in the mesentery. Yellow bullheads in this pond had been stocked from a river and were dying from an undetermined cause, whereas black bullheads, stocked from another source, seemed to be thriving and harbored no parasites.

Metacercariae of *Clinostomum marginatum* make the flesh of fish undesirable for consumption, but evidently do the host no severe harm.

The intestinal parasites seem to be well adapted to their hosts and, except for the acanthocephalans, cause no evident pathology.

The leech, *Placobdella montifera,* caused severe hemorrhage around the site of attachment to the host and might conceivably be detrimental due to secondary bacterial infections.

Copepods and monogenetic trematodes caused no apparent pathology to the gill filaments or gill arches despite the large number of parasites generally present.

Hubbs (1927) studied the effects of parasites on the cyprinid, *Hybopsis* (=*Platygobio*) *gracilis,* and found that parasitism retarded early growth, caused the retention of larval characters, and increased the number of scales. No comparable work has been done with catfishes.

SUMMARY AND CONCLUSIONS

In 1956 and 1957, 135 catfishes of the species *Ictalurus punctatus, I. melas,* and *I. natalis* were collected, representing all months except November, December, and January, from 12 ponds, 2 lakes, 3 rivers, and 5 creeks of northeastern Kansas.

One hundred twenty-four (91.9 percent) of the fish were parasitized by at least one species. Twenty-four species of trematodes, cestodes, acanthocephalans, nematodes, leeches, and copepods were collected.

The degree of parasitism was affected by ecological habitat of host and season of year more than by size of the host. The peak of infection occurred in June, July, and August, during which time 90 to 100 percent of the fish examined were parasitized.

External parasites (leeches, copepods, and monogenetic trematodes) were significantly fewer in ponds than in lakes and streams.

The gut harbored 44.2 percent of all the parasites found; 43 percent were attached to the gills; 1.2 percent were found in the urinary bladder; 0.1 percent were attached to the surface of the skin; and 10.7 percent were encysted in the viscera and mesenteries.

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RESEARCH NOTE

SOME BLOOD PARASITES FOUND IN BIRDS AND MAMMALS FROM LOEI PROVINCE, THAILAND.

While working with a malaria control unit of the United States Operations Mission to N. E. Thailand in 1954 and 1955, the junior authors collected several hundred blood smears from birds and mammals of Loei province. The smears were stained with Giemsa in the field and later submitted to the senior author for study. Skins of the birds and mammals were deposited in the U. S. National Museum, Washington, D. C. where identifications were made by H. G. Deignan (birds) and Dr. David H. Johnson (mammals) to whom thanks are extended.

The parasites found in the blood of this group of animals are listed in table I with identification, for the most part, limited to genus. Several factors contributed to this decision, the most important being: (1) The lack of sufficient material, in some cases only one slide, and (2) considerable reluctance to assign or recognize specific names when most of the evidence rests on host differences rather than morphological or experimental evidence.

Haemoproteus. This genus was observed in 25 different species of birds. Twenty-three species harbored parasites of the *H. columbae*-type. Among this group, three species (the white-winged woodduck, the banded kingfisher, and the blue-throated barbet) were parasitized by a more rotund *H. columbae*-type halteridium which crowded the host cell nucleus close to the cell wall. Two species exhibited parasites of the *H. velans*-type (Coatney and Roudabush, 1937, Am. Midl. Nat. **18:** 1005–1030). Huff (1950, J. Nat. Mal. Soc. **9:** 307–319), in discussing *Plasmodium fallax*, points out that infections with that species tend to become chronic with mostly gametocytes in the blood. He also says that *fallax* gametocytes are indistinguishable from the gametocytes of *Haemoproteus* and consequently many records of halteridium parasites from wild birds are suspect especially if the diagnosis was made from a single blood smear. The difficulty of diagnosis is well recognized in the present study, but in the absence of distinguishing criteria, all halteridium-like parasites have been designated as *Haemaproteus*.

Leucocytozoon. Parasites of this genus were found in 15 different species of birds. Thirteen species had parasites of the round type and two species (the red jungle fowl and the lesser long-billed thrush) had spindle-form parasites.

Plasmodium. Malaria parasites were found in three different species of squirrels. All of these parasites were mature circular gametocytes which did not appear to increase the size of the host cell. There were no recognizable differences between the parasites from the different hosts. In an attempt to determine the species, comparison was made with two named species from small mammals—*Plasmodium vassali* (Laveran, 1905) from *Sciurus griseimanus* (yellow-handed squirrel) and *P. brodeni* Rodhain, Pons, Vandenbranden, and Bequaret, 1913 from *Petrodromus tetradactylus* (forest elephant shrew). Slides of *P. brodeni* from the African elephant shrew, *Elephantulus rufescens dundasi* were kindly supplied by Dr. C. G. Huff and it was found that the parasite in question closely resembled *P. brodeni* except that the mature macrogametocytes do not produce hypertrophy of the host cells. When Dr. Huff examined the material he concurred in the above opinion. It should be mentioned, however, that since *P. vassali* also comes from a squirrel, our material, which resembles *vassali* could possibly belong to that species. Until further evidence is available, the taxonomic status of this *Plasmodium* is in doubt. None of the birds harbored parasites that could be identified as plasmodia.

Trypanosoma. Parasites of this genus were found in only one bird; all specimens were without myonemes. Four of the mammals harbored trypanosomes and all were of the *T. lewisi*-type.

Microfilaria. Microfilariae were found in 27 different species of birds and 3 species of mammals.

Considering all infections, there were nine double infections, six triple infections and one quadruple infection.—G. ROBERT COATNEY, *Laboratory of Parasite Chemotherapy, National Institutes of Health, U. S. Department of Health, Education, and Welfare, Public Health Service, Bethesda, Md.;* ROBERT E. ELBEL, *Department of Zoology, University of Oklahoma, Norman, Oklahoma, and* PRIDAR KOCHARATANA, *Division of Malaria Control, Thailand Department of Health.*

TABLE I. *Blood parasites found in birds and mammals from Loei Province, Thailand. (Host names under Aves and Mammalia are listed in phylogenetic order.)*

Scientific name	Common name	Number		Haemoproteus	Leucocytozoon	Plasmodium	Trypanosoma	Microfilaria
		Examined	Infected					
AVES								
Ardeola ralloides bacchus (Bonaparte)	Chinese pond heron	4	3					X
Cairina scutulata (S. Müller)	white-winged woodduck	1	1	C				
Aviceda leuphotes leuphotes (Dumont)	black-crested baza	1	1					X
Gallus gallus spadiceus (Bonnaterre)	red jungle fowl	8	5		Sp			X
Turnix suscitator thai Deignan	barred button quail	1	1					X
Scolopax rusticola rusticola Linnaeus	woodcock	1	1	V				
Ducula badia griseicapilla Walden	mountain imperial pigeon	6	1	C				
Macropygia unchall tusalia (Blyth)	barred-tailed cuckoo dove	3	1	C				
Macropygia ruficeps assimilis Hume	rufous-headed cuckoo dove	2	1	C				
Streptopelia orientalis agricola (Tickell)	rufus dove	5	1	C				X
Psittacula alexandri fasciata (P.L.S. Müller)	pink-breasted parakeet	2	1	V				
Phaenicophaeus tristis longicaudatus Blyth	large green-billed malkoha	12	1					X
Otus bakkamoena lettia (Hodgson)	collared scops owl	1	1	C				X
Glaucidium brodiei brodiei (Burton)	collared pygmy owl	1	1	C				
Glaucidium cuculoides brügeli (Parrot)	barred pygmy owl	3	3	C	R			X
Harpactes oreskios stellae Deignan	orange-breasted trogon	1	1	C				
Lacedo pulchella amabilis (Hume)	banded kingfisher	1	1	C				
Nyctyornis athertoni athertoni (Jardine and Selby)	blue-bearded bee-eater	5	2					X
Anthracoceros albirostris leucogaster (Blyth)	pied hornbill	4	1		R			X
Megalaima virens virens (Boddaert)	giant barbet	4	1	C				
Megalaima zeylanica hodgsoni Bonaparte	lineated barbet	2	1	C				
Megalaima asiatica laurentii (Wells)	blue-throated barbet	11	9	C				X
Picus canus hessei Gyldenstolpe	black-naped green woodpecker	3	1	C				
Chrysocolaptes lucidus guttacristatus (Tickell)	four-toed golden-backed woodpecker	9	1					X
Psarisomus dalhousiae assimilis Hume	long-tailed broadbill	2	2	C				X
Pitta oatesi oatesi (Hume)	rufous-naped pitta	2	1		R			
Pericrocotus flammeus semiruber Whistler and Kinnear	scarlet minivet	8	2	C				
Coracina novachollandiae siamensis (Stuart Baker)	ashy cuckoo-shrike	5	2					X
Dicrurus leucophaeus mouhoti (Walden)	pale ashy drongo	2	1					X
Dicrurus annectens (Hodgson)	crow-billed drongo	1	1					X
Dicrurus aeneus aeneus Vieillot	bronzed drongo	2	1	C	R			X
Oriolus chinensis diffusus Sharpe	black-naped oriole	1	1		R			
Corvus macrorhynchos levaillantii Lesson	jungle crow	1	1	C	R			X
Garrulus glandarius leucotis Hume	white-eared jay	6	5	C	R			X
Kitta chinensis chinensis (Boddaert)	green-breasted hunting crow	1	1					X
Crypsirina formosae himalayana (Jerdon)	gray tree pie	4	3					X
Irena puella puella (Latham)	fairy blue bird	4	1					X
Pycnonotus aurigaster klossi (Gyldenstolpe)	red-vented bulbul	2	1		R			
Pycnonotus melanicterus auratus Deignan	crested yellow bulbul	1	1	C	R			
Criniger pallidus isani Deignan	white-throated bulbul	11	4	C	R			X
Hypsipetes madagascariensis concolor Blyth	black bulbul	4	1		R			
Hypsipetes madagascariensis leucothorax (Mayr)	white-headed black bulbul	2	1		R			
Garrulax chinensis lochmius Deignan	black-throated laughing thrush	3	2					X
Yuhina zantholeuca tyrannulus (Swinhoe)	white-bellied yuhina	2	1	C				
Enicurus leschenaulti indicus Hartert	white-crowned forktail	1	1					X
Zoothera marginata Blyth	lesser long-billed thrush	3	2		Sp			X
Monarcha azurea montana (Riley)	black-naped flycatcher	1	1				X	
Gracula religiosa intermedia Hay	grackle	14	8	C	R			X
MAMMALIA								
Tupaia belangeri laotum Thomas	tree shrew	19	2				L	
Ratufa bicolor gigantea McClelland	giant squirrel	5	1			X		
Callosciurus finlaysoni subsp. (near *bocourtii*)	tree squirrel	112	8			X	L	X
Tamiops mcclellandii kongensis Bonhote	striped tree squirrel	63	15			X	L	
Menetes berdmorei consularis Thomas	striped ground squirrel	13	1					X
Petaurista lylei subsp. (probably near *badiatus*)	large flying squirrel	1	1					X
Rattus rattus subsp. (near *thai*)	field rat	9	1				L	

C, columbae-type; L, lewisi-type; R, round-type; Sp, spindle-type; V, velans-type.

STUDIES ON THE HELMINTH FAUNA OF ALASKA. XXXVIII. THE TAXONOMIC SIGNIFICANCE OF EGGS AND CORACIDIA OF SOME DIPHYLLOBOTHRIID CESTODES

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Diphyllobothriid cestodes often lack dependable taxonomic characters in the strobilar stage and consequently may be difficult if not impossible to identify. This is particularly true for species of *Diphyllobothrium* Cobbold, 1858, which are rather uniform in structure and yet may be subject to morphological variation evidently dependent on host and other factors (Rausch, 1954). It is desirable, therefore, to determine whether other stages in the life cycle possess characteristics of taxonomic value.

This paper reports findings on 11 species of diphyllobothriid cestodes collected in Alaska. A detailed study of the eggs and coracidia was undertaken to investigate their possible taxonomic significance. The following characteristics of the eggs were considered: size, shape, thickness of the shell, and design on its surface, and the presence or absence of an apical knob. Characteristics of the coracidia that were considered include: size and form of the embryonic hooks, numbers of plastin cells, and, in the living coracidia, rate and pattern of swimming and response to variations in temperature and salinity. Observations were made also on the response to light and temperature, and the effect of salinity and pressure on the hatching and behavior of coracidia.

Particular emphasis was placed upon the possible significance of numbers of plastin cells as a taxonomic character at the species level. These structures were regarded as germinal cells by Vogel (1929) and Kuhlow (1953), but more recent studies by Silverman (1954) indicate that this is not the case.

MATERIALS AND METHODS

Eggs were collected from cestodes obtained at the autopsy of both experimentally and naturally infected birds and mammals. Material from man was obtained through the use of anthelminthic drugs, primarily atabrine.

Eggs were available from the following species: *Diphyllobothrium ?dendriticum* (Nitzsch, 1824); *D. ditremum* (Creplin, 1825); *D. cordatum* (Leuckart, 1863); *D. lanceolatum* (Krabbe, 1865); *D. osmeri* (von Linstow, 1878); *D. ursi* Rausch, 1954; *D. dalliae* Rausch, 1956; *D. alascense* Rausch and Williamson, 1958; *Diplogonoporus balaenopterae* Lönnberg, 1892; *Pyramicocephalus phocarum* (Fabricius, 1780), and *Schistocephalus solidus* (Müller, 1776). Also included was *Diphyllobothrium* sp. indet.; although its identity has not been established, it can be distinguished from the other species by characteristics of the coracidium. The initial determinations of these cestodes were made by Dr. Robert Rausch, of this Center, and all other identifications were reviewed by him.

After being released from the uterine coils by dissection, the eggs were washed several times in fresh water and stored in water at 2 C; in most cases, they would survive as long as 4 months at this temperature. The eggs were measured and counted in a nannoplankton chamber (Palmer, 1954) in order to avoid any distortion from the pressure of the cover glass. Only eggs containing embryos were utilized, since it was noted that those not normally developed often varied in size.

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Plastin cells were stained by the methyl green-pyronin method of Vogel (1929), although it was found necessary to reduce the staining time to a maximum of 15 minutes in order to avoid overstaining of coracidia from cestodes taken from marine mammals. Because of its higher viscosity, human serum was found superior to that available from other mammals for attaching the coracidia to slides.

The coracidium is bilaterally symmetrical, with three embryonic hooks on each side. For convenience, these have been numbered from 1 to 3 on each side, designated "right" and "left"; number 1 being the outermost on each side, number 2 the middle hook, and number 3 the hooks of the middle pair (see fig. 1, A). Hooks 1, 2, and 3 differ from one another morphologically.

Methods for determining the effects of salinity on coracidia and hydrostatic pressure on hatching of eggs are described in a later section.

I. Observations on Eggs and Coracidia

(All measurements in microns unless otherwise indicated)

(1) *Diphyllobothrium ?dendriticum* (Nitzsch, 1824)

Source of material: Dog, *Canis familiaris* L. (experimental infection); glaucous-winged gull, *Larus glaucescens* Naumann (natural infection) Karluk Lake, Kodiak; and herring gull, *Larus argentatus* Pontoppidan (experimental infection). Last intermediate host: *Salmo gairdnerii* Richardson, in lakes of north-central Kenai Peninsula. The identification of this cestode is tentative pending study of additional material.

Eggs from strobilae from 2 herring gulls and 1 glaucous-winged gull had following characteristics: broadly ovoid in shape, symmetrical; shell about 1.0 thick, amber in color, smooth. Coracidium situated near opercular end. Operculum with average diameter of 18.8; sutures not incised. Apical knobs usually conspicuous. Measurements are given in table I.

Coracidia within shells averaged 32 by 35; after hatching, they averaged 38 by 41.6. Incubation period 6 to 8 days in fresh water at 20 C. Coracidia active up to 18 hours at 20 C; up to 4 days at 20 C. Anterior cilia averaged 33 long; posterior cilia averaged 10. In fresh water at 20 C, newly hatched larvae swam 1 mm in 6 seconds. No embryonation in physiological saline.

Embryonic hooks (fig. 1, B) had following average lengths: (1) R 11.30, L 11.00; (2) R 12.40, L 12.10; (3) R 12.5, L 12.40. Hooks similar to those of *D. alascense* in shape.

Data on plastin cells, representing strobilae from an experimentally infected herring gull and a naturally infected glaucous-winged gull, are shown graphically in figure 2, X–Z, and numerically in table II.

(2) *Diphyllobothrium ditremum* (Creplin, 1825)

Source of material: Two loons, *Gavia immer* (Brunnich) (natural infection), from lakes in north-central Kenai Peninsula.

Eggs small, obovoid; greatest diameter near apical knob. Amber-colored shells about 1.2 thick; average diameter of operculum 20.5; sutures not incised. Coracidium usually situated near operculate end of egg. Measurements of eggs are given in table I.

Coracidia elongate-ovoid, averaging 28 by 32. Hatching occurred on ninth day in fresh water at 20 C. Upon hatching, coracidia swam vertically to surface where they remained relatively stationary for few minutes; thereafter they sank to bottom of dish where they moved feebly about. Survival in fresh water at 20 C was less than 10 hours; at 2 C they survived 3 days. The eggs would not embryonate in physiological saline.

Embryonic hooks (fig. 1, C) had following average lengths: (1) R 10.43, L 10.71; (2) R 12.17, L 12.04; (3) R 12.60, L 12.47. Shaft, blade, and guard noticeably robust in all hooks. Shape of hook no. 3 had greatest diameter of any examined in present study, usually exceeding 1; also, blade of this hook had strongly curved tip.

Numbers of coracidia were insufficient for study of plastin cells.

(3) *Diphyllobothrium cordatum* (Leuckhart, 1863)

Source of material: Bearded seal, *Erignathus barbatus* (Erxleben) (natural infection), St. Lawrence Island; dog (natural infection), Tanunak.

In cestodes from two bearded seals and from a dog, eggs symmetrical, characteristically long and narrow (width: length ratio 1: 1.5), with greatest diameter near middle. Shell 3 thick; dark amber in color, and scrobiculate (pits about 0.54 in diameter) (pl. I, fig. 5). Elongate-ovoid coracidium situated centrally within egg. Average diameter of operculum 21; sutures strongly incised. Apical knob lacking. Measurements of eggs are given in table I.

Coracidia within shells averaged 34 by 41; after emergence in physiological saline, they averaged 41 in diameter. Survival time in physiological saline at 20 C was 5 to 6 days; the following observations also were made: (1) Eggs in tap water embryonated but did not hatch;

TABLE I. *Statistical analyses of egg measurements for cestodes listed below.*

No. eggs	Host	Mean width & length	Range width	Range length	Std. dev. width	Std. dev. length	Ratio width/ length
			Diphyllobothrium dendriticum				
50	Herring Gull	40.7–58.0	35.7–42.8	54.7–60.7	1.84	1.64	1 : 1.43
50	Herring Gull	43.3–58.0	40.4–45.2	51.2–61.8	1.05	2.47	1 : 1.34
50	Glaucous-winged Gull	42.4–60.0	41.6–44.0	55.9–65.4	1.00	1.95	1 : 1.42
			Diphyllobothrium ditremum				
50	Common Loon	39.8–53.8	39.3–41.6	51.2–55.9	1.35	2.96	1 : 1.35
50	Common Loon	37.2–53.3	34.5–41.6	48.8–61.9	1.73	3.15	1 : 1.43
			Diphyllobothrium cordatum				
50	Bearded Seal	42.4–64.4	39.3–44.0	59.5–69.0	1.24	2.55	1 : 1.52
50	Bearded Seal	41.8–66.3	40.5–42.8	64.3–71.4	0.70	1.83	1 : 1.58
50	Bearded Seal	42.0–65.4	40.5–44.0	60.7–69.0	0.69	1.96	1 : 1.56
50	Dog	42.9–70.1	41.6–44.0	64.5–75.0	0.71	3.64	1 : 1.63
			Diphyllobothrium lanceolatum				
50	Bearded Seal	45.6–62.4	42.8–47.6	55.9–70.2	1.61	2.19	1 : 1.37
50	Bearded Seal	42.7–58.5	40.5–45.2	54.7–60.7	1.10	1.58	1 : 1.37
50	Bearded Seal	44.3–65.6	42.8–46.4	63.1–70.2	1.65	0.94	1 : 1.48
50	Bearded Seal	42.0–70.7	42.8–45.2	58.3–66.6	0.65	2.15	1 : 1.65
			Diphyllobothrium osmeri				
50	Dog	42.4–60.5	40.5–52.4	45.2–66.6	1.27	2.45	1 : 1.43
			Diphyllobothrium ursi				
50	Brown Bear	43.7–66.3	40.5–47.6	60.7–74.9	1.62	3.36	1 : 1.52
50	Brown Bear	43.4–59.9	40.4–46.4	55.9–65.4	1.82	2.66	1 : 1.38
50	Brown Bear	39.0–57.5	36.9–41.6	51.2–64.2	1.21	2.75	1 : 1.47
50	Brown Bear	44.5–64.8	40.4–47.6	59.5–71.3	1.93	3.03	1 : 1.46
50	Brown Bear	44.3–61.0	39.3–47.6	53.5–66.6	2.12	3.02	1 : 1.37
50	Glaucous-winged Gull	40.7–55.0	35.7–42.8	50.0–59.5	1.87	2.19	1 : 1.35
			Diphyllobothrium dalliae				
50	Glaucous-winged Gull	43.0–59.1	38.1–45.2	48.8–64.2	1.53	4.12	1 : 1.37
50	Man	50.3–66.5	47.6–53.5	61.9–71.4	1.44	3.08	1 : 1.30
50	Man	49.6–67.3	44.0–53.5	58.3–72.6	2.38	3.59	1 : 1.35
			Diphyllobothrium alascense				
50	Dog	47.0–67.6	38.1–57.1	57.1–71.4	3.44	3.50	1 : 1.44
50	Dog	46.8–65.9	41.6–58.3	57.1–79.7	3.56	5.40	1 : 1.40
50	Dog	47.8–64.3	41.6–55.9	59.5–71.4	4.00	3.90	1 : 1.34
50	Dog	48.5–73.4	42.8–66.6	55.9–78.6	—	—	1 : 1.51
			Diplogonoporus balaenopterae				
50	Dog	47.3–75.4	44.0–51.2	65.5–82.1	1.48	3.50	1 : 1.60
			Pyramicocephalus phocarum				
50	Bearded Seal	40.7–61.3	39.3–42.8	58.3–64.3	1.76	0.95	1 : 1.50
50	Bearded Seal	40.8–63.0	38.1–42.8	58.3–67.8	2.52	1.23	1 : 1.54
50	Bearded Seal	39.9–60.8	36.9–41.7	58.3–64.3	1.61	1.22	1 : 1.52
			Schistocephalus solidus				
50	Dog	49.3–71.5	44.0–52.4	64.4–77.3	1.94	3.68	1 : 1.45
50	Dog	47.7–71.6	44.0–51.2	65.4–78.5	2.16	4.00	1 : 1.50

(2) in 1.5 percent saline, most eggs embryonated but only about 50 percent hatched; (3) all coracidia hatched and swam about in 3 to 3.4 percent saline; (4) in 4.2 percent saline, many emerged but few swam; (5) in 5 and 7 percent saline, embryonation occurred but only 4 and 2 percent hatched, respectively; (6) in 8 percent saline, most eggs embryonated but the coracidia died when about half emerged through the opercular opening. In sea water (3.23 percent saline) coracidia survived 104 days at 2 C; at 20 C they survived only 2 days.

Embryonic hooks (fig. 1, D) had the following average lengths: (1) R 10.77, L 10.23; (2) R 11.84, L 11.07; (3) R 12.38, L 13.10. Hooks nos. 2 and 3 possess massive guards extending at right angles from long axes of shafts; these are characteristic for *D. cordatum*.

Data on plastin cells, representing strobilae from two naturally infected bearded seals and a naturally infected dog, are shown graphically in figure 2, A–C, and numerically in table II.

(4) *Diphyllobothrium lanceolatum* (Krabbe, 1865)

Source of material: Two bearded seals (natural infection), St. Lawrence Island.

Ovoid eggs longer than wide, with greatest diameter at center; shell 2.5 thick, amber to dark amber in color, with scrobiculate surface (pits about 0.50 in diameter) (pl. I, fig. 4). Operculum averaged about 19 in diameter; sutures strongly incised. Coracidium centrally located.

Coracidia within shells averaged 30 by 41; after emerging in physiological saline, they averaged 42 in diameter. In physiological saline at 20 C, incubation period 12 to 18 days.

Posterior cilia averaged 8.3 long; anterior cilia averaged 14.3.

Embryonic hooks (fig. 1, E) had following measurements: (1) R 11.42, L 11.26; (2) R 11.80, L 12.23; (3) R 14.22, L 14.31.

Coracidia were too few to permit study of plastin cells.

(5) *Diphyllobothrium osmeri* (von Linstow, 1878)

Source of material: Dog (experimental infection). Last intermediate host: *Osmerus dentex* Steindachner, from Naknek River near King Salmon.

Eggs distinctly obovoid; shell usually 1.2 thick, smooth, and pale yellow in color. Apical knob inconspicuous. Average diameter of operculum 22.4; sutures not incised. Coracidium situated centrally within egg.

Coracidia measured about 37 within the shells, and 40 after emergence. Incubation period 11 days in fresh water at 20 C. In latter, coracidia swam 1 mm in 4 seconds. Eggs did not embryonate in physiological saline. Coracidia very fragile; cilia not measured.

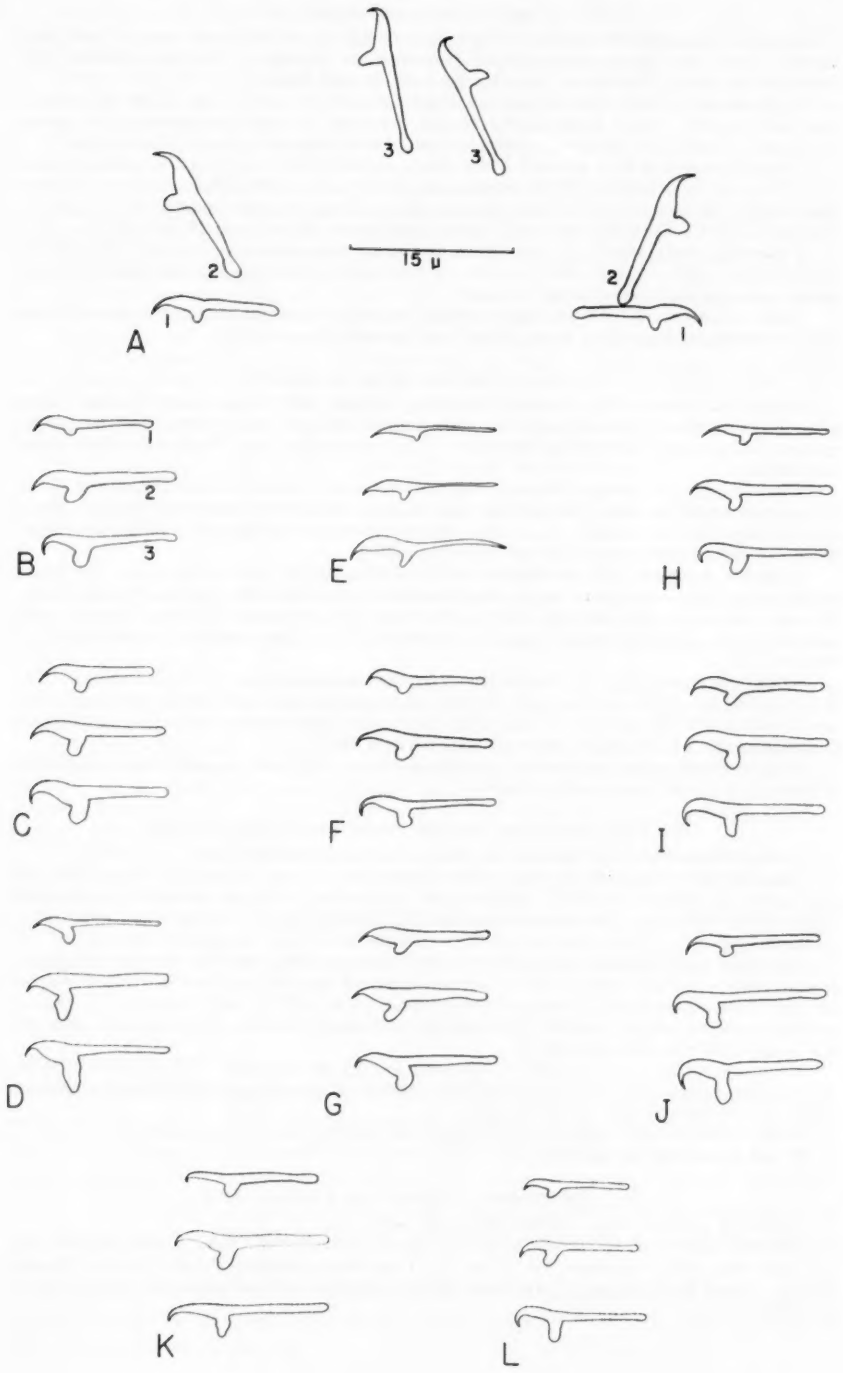
TABLE II. *Statistical analyses of plastin cells for the cestodes listed below.*

No. coracidia	Host	Range	Mean	Std. dev.	Std. error	Coefficient variation, percent
		Diphyllobothrium ?dendriticum				
200	Herring Gull	1–10	5.10	1.70	0.12	33.3
200	Herring Gull	1–10	5.27	1.51	0.10	28.6
200	Glaucous-winged Gull	1–9	4.96	1.35	0.096	27.2
		Diphyllobothrium cordatum				
376	Bearded Seal	12–26	18.69	2.30	0.118	12.3
266	Bearded Seal	11–25	17.90	2.93	0.195	16.3
210	Dog	11–25	18.39	2.89	0.20	15.7
		Diphyllobothrium osmeri				
403	Dog	6–19	11.03	2.09	0.145	18.84
400	Dog	4–17	10.70	1.92	0.096	17.95
		Diphyllobothrium ursi				
400	Brown Bear	3–13	7.97	1.83	0.091	22.9
389	Brown Bear	3–13	8.31	1.89	0.096	22.7
		Diphyllobothrium dalliae				
180	Man	3–19	10.17	2.70	0.20	26.50
403	Arctic Fox	3–18	10.18	2.30	0.115	22.60
210	Man	3–16	9.98	2.10	0.145	21.00
124	Man	4–16	10.06	2.23	0.187	22.20
143	Man	4–16	10.09	2.26	0.203	22.40
400	Dog	3–15	9.83	1.95	0.098	19.80
		Diphyllobothrium alascense				
97	Dog	2–10	5.90	2.10	0.21	35.6
430	Dog	2–11	5.85	1.52	0.12	27.0
209	Dog	1–11	5.87	1.41	0.10	24.0
109	Dog	1–11	5.78	1.87	0.18	32.3
		Pyramicocephalus phocarum				
180	Bearded Seal	10–24	17.09	2.65	0.20	15.5
180	Bearded Seal	10–24	16.64	2.88	0.21	17.3
		Schistocephalus solidus				
400	Dog	7–18	11.88	2.11	0.11	17.7
400	Dog	5–17	11.90	2.08	0.10	17.5
400	Grebe	4–17	10.82	2.33	0.11	21.5
400	Grebe	4–17	11.32	2.44	0.12	21.5

Embryonic hooks (fig. 1, F) had following average lengths: (1) R 10.72, L 11.12; (2) R 12.31, L 12.40; (3) R 12.55, L 12.47. Notch at posterior margin of guard appears characteristic of *D. osmeri*.

Data on plastin cells, representing strobilae from one experimentally infected dog, are shown graphically in figure 2, J–K, and numerically in table II.

FIGURE 1. A, typical arrangement of embryonic hooks within a coracidium. As numbered, hooks 1, 2, and 3 are antipodes of 3, 2, and 1; members of each pair are identical. B, *Diphyllobothrium ?dendriticum;* C, *D. ditremum;* D, *D. cordatum;* E, *D. lanceolatum;* F, *D. osmeri;* G, *D. ursi;* H, *D. dalliae;* I, *D. alascense;* J, *Diplogonoporus balaenopterae;* K, *Pyramicocephalus phocarum;* L, *Schistocephalus solidus.*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(6) *Diphyllobothrium ursi* Rausch, 1954

Source of material: Brown bear, *Ursus arctos middendorfii* Merriam (natural infection), Kodiak Island; and glaucous-winged gull (experimental infection). Last intermediate host: *Oncorhynchus nerka* (Walbaum), from Karluk Lake, Kodiak Island.

Eggs obovoid with greatest diameter near opercular end; shell 1.2 thick, amber to brown in color, and smooth. Apical knobs usually present. Average diameter of operculum 20; sutures not incised. Coracidium situated at opercular end. Dimensions of eggs are given in table I.

Coracidia averaged 36 in diameter within shells, and 46.8 after emergence. Incubation period 14 to 19 days in fresh water at 20 C; embryonation did not occur in physiological saline. Anterior cilia averaged 35.7 long; posterior cilia averaged 23.8. Newly emerged coracidia swam 1 mm in 6 seconds at 20 C. Coracidia survived 8 days in fresh water at 20 C, and 27 days at 2 C.

Embryonic hooks (fig. 1, G) had following average measurements: (1) R 11.90, L 11.80; (2) R 13.10, L 12.80; (3) R 12.90, L 12.90. In hook no. 3, anteriorly directed, bulb-like guard appears constant and characteristic of *D. ursi*.

Data on plastin cells, representing strobilar fragments from one naturally infected brown bear, are shown graphically in figure 2, R–S, and numerically in table II.

(7) *Diphyllobothrium dalliae* Rausch, 1956

Source of material: Man (natural infection), Chevak, Mt. Village, and Tanunak; arctic fox, *Alopex lagopus* L. (natural infection), St. Lawrence Island; dog (experimental infection); glaucous-winged gull (experimental infection). Last intermediate host: Black fish, *Dallia pectoralis* Bean.

Obovoid eggs with greatest diameter near opercular end. Shell 1.2 thick, smooth (pl. I, fig. 1), and pale amber in color. Diameter of operculum averaged 21.4; sutures not incised. Apical knob inconspicuous or lacking. Coracidium situated near center of egg. Measurements of eggs from man and glaucous-winged gull are shown in table I.

Coracidia averaged 41.6 in diameter within shells, and 48 after emergence. Incubation period varied from 7 to 8 days (man, glaucous-winged gull, and arctic fox) to 19 days (dog). No eggs hatched in physiological saline. Posterior cilia averaged 17.4 long; anterior cilia averaged 31.5. Coracidia swam 1 mm in 8 seconds at 20 C. They survived 2 days at 20 C; 5 days at 2 C.

Embryonic hooks (fig. 1, H) had following average measurements: (1) R 11.30, L 11.60; (2) R 12.87, L 13.30; (3) R 12.22, L 12.05. In hook no. 3, slender shaft with robust guard and blade seem constant and characteristic of *D. dalliae*. Coracidia from cestodes obtained from dog were anomalous, with 2 to 11 hooks; many of these had split shafts.

Data on plastin cells, representing strobilae from man, dog, and fox, are shown graphically in figure 2, L–Q, and numerically in table II.

(8) *Diphyllobothrium alascense* Rausch and Williamson, 1958

Source of material: Dog (natural infection), Chevak and Hooper Bay.

Obovoid eggs comparatively long (width: length ratio 1: 1.4). Shell 2.15 to 2.4 thick and light amber to yellowish in color; surface finely scrobiculate, with pits about 0.46 in diameter (pl. I, fig. 2). Average diameter of operculum 19.5; sutures incised. Apical knobs inconspicuous or lacking. Coracidium situated centrally. Dimensions of eggs are given in table I.

Coracidia within shells averaged 28 by 45; they measured 36 by 40 after emerging in physiological saline. In fresh water at 20 C, incubation period was 10 days, but less than 1 percent hatched. Coracidia survived 12 hours in physiological saline at 20 C, and 2 days at 2 C. Newly emerged coracidia swam 1 mm in 33 seconds in physiological saline. Posterior cilia averaged 8 in length; anterior cilia averaged 26.

Embryonic hooks (fig. 1, I) had the following average measurements: (1) R 11.40, L 10.50; (2) R 12.80, L.12.20; (3) R 12.50; L 12.80. In hook no. 3, strongly curved and finely attenuated blade appears characteristic of this species.

Data on plastin cells, representing strobilae from one dog, are shown graphically in figure 2, T–W, and numerically in table II.

(9) *Diplogonoporus balaenopterae* Lönnberg, 1892

Source of material: Dog (natural infection), Anchorage.

Obovoid eggs elongate and narrow (width: length ratio 1: 1.6). Shell 2 thick, scrobiculate, with pits about 0.86 in diameter (pl. I, fig. 6). Operculum averaged 21.4 in diameter; sutures incised. Apical knob lacking. Coracidium situated centrally. Measurements of eggs are given in table I.

Coracidia within shells measured 43.4 by 48.5. Eggs embryonated in salinities ranging from 0 to 3.5 percent, but failed to hatch after 7 days at 20 C. By changing saline concentrations from higher to lower levels, coracidia were expelled but embryophores ruptured.

Embryonic hooks (fig. 1, J) had the following average measurements: (1) R 12.16, L 11.30; (2) R 13.90, L 13.92; (3) R 13.04, L 13.24. In hooks nos. 2 and 3, bulbous guards protruding at right angles appear to be typical.

Coracidia were too few to permit study of plastin cells.

(10) *Pyramicocephalus phocarum* (Fabricius, 1780)

Source of material: Bearded seal (natural infection), St. Lawrence Island. Last intermediate host: Cod, *Eleginus gracilis* (Tilesius).

Elongate eggs narrowly obovoid, with greatest diameter near operculate end; shell about 2.5 thick, amber in color, and scrobiculate, with pits about 0.40 in diameter. Average diameter of operculum 19.5; sutures strongly incised. Apical knob much reduced or lacking. Coracidium situated centrally. Measurements of eggs are given in table I.

Coracidia within shells averaged 35 by 38; after emerging in physiological saline, they averaged 35.7 in diameter. Eggs did not embryonate in fresh water. In physiological saline at 20 C, incubation period was 9 to 12 days. Coracidia survived in sea water (3.23 percent salt) 4 days at 20 C, and 30 days at 2 C. Best hatching success was noted in salinities of 2.5 to 3 percent; lower or higher concentrations produced fewer coracidia. Posterior cilia averaged 7.2 in length; anterior cilia averaged 30.

Embryonic hooks (fig. 1, K) had following average measurements: (1) R 11.84, L 11.30; (2) R 15.07, L 15.76; (3) R 13.72, L 13.45.

Data on plastin cells, representing strobilae from two bearded seals, are shown graphically in figure 2, D–E, and numerically in table II.

(11) *Schistocephalus solidus* (Müller, 1776)

Source of material: Red-necked grebe, *Podiceps grisegena* Reinhardt (natural infection), lakes in north-central Kenai Peninsula; dog (natural infection), Kipnuk and Newtok. Last intermediate host: 3-spined stickleback, *Gasteriosteus aculeatus* Girard, and 9-spined stickleback, *Pungitius pungitius* (L).

Large, asymmetrical eggs pointed at opercular end. Shell only 1.2 thick, smooth, and pale yellow in color. Apical knob absent; average diameter of operculum about 16. Coracidium situated centrally. Dimensions of eggs are presented in table I.

Coracidia within shells averaged 38 by 42. Incubation time in both fresh water and physiological saline at 20 C was 14 to 16 days. Emerged coracidia averaged 43 by 47 in fresh water, and 40 by 45 in physiological saline. Freshly emerged coracidia swam 1 mm in 1 second and showed strong directional movement. Survival in fresh water at 20 C was 8 days; at 2 C, 2 days. Survival time in normal saline at 20 C was 3 days; at 2 C, 2 days.

Posterior cilia averaged 13.5 in length; anterior cilia averaged 21.5. Cilia exhibited wavelike motion proceeding from anterior to posterior end; this was not observed in coracidia of other species studied.

Embryonic hooks (fig. 1, L) had following average measurements: (1) R 8.62, L 8.32; (2) R 10.70, L 10.32; (3) R 11.30, L 11.80.

Data on plastin cells, representing strobilae from two grebes and two dogs, are shown graphically in figure 2, F–I, and numerically in table II.

II. General Biological Observations

Salinity and Isotonicity

Previous workers have reported that when coracidia of *Diphyllobothrium* spp. hatch in fresh water there is a pronounced swelling of the embryophore, due presumably to osmotic phenomena (Kuhlow, 1953; Vik, 1957). Further, it has been noted that coracidia expand more slowly and survive for longer periods in colder water. In coracidia of marine origin, however, the writer found little evidence of swelling of the embryophore, indicating that its fluids were already isotonic with the sea water.

An attempt to evaluate the differential effects of salinities on the embryophore utilized freshly hatched coracidia from cestodes whose larvae apparently live under different conditions of salinity.

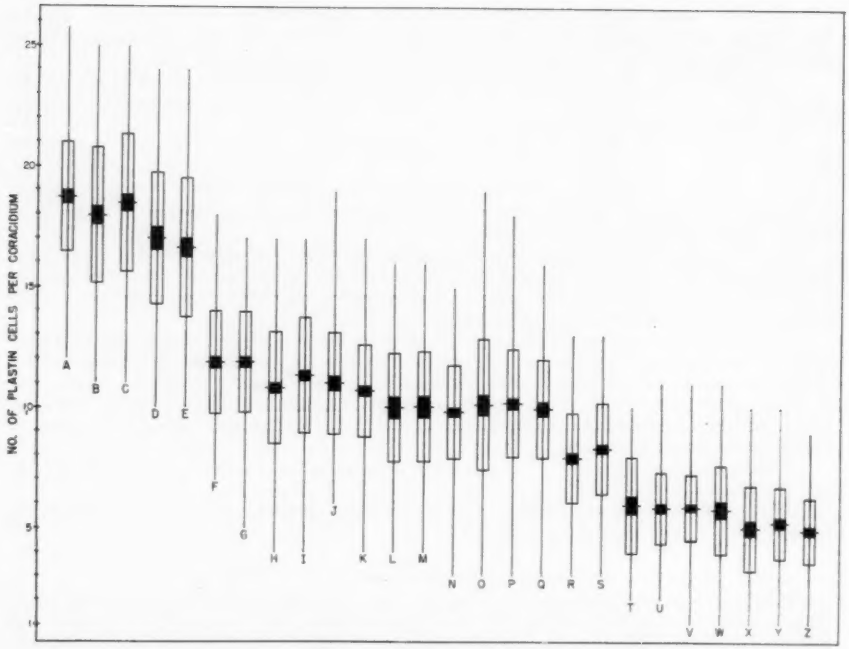

FIGURE 2. Statistical analyses of plastin cell counts for the cestodes listed below. Vertical lines indicate range, open rectangles mark standard deviation, and solid black rectangles show twice the standard error of the mean (horizontal line). A, *Diphyllobothrium cordatum ex* bearded seal; B, *D. cordatum ex* bearded seal; C, *D. cordatum ex* dog; D, *Pyramicocephalus phocarum ex* bearded seal; E, *P. phocarum ex* bearded seal; F, *Schistocephalus solidus ex* dog; G, *S. solidus ex* dog; H, *S. solidus ex* red-necked grebe; I, *S. solidus ex* red-necked grebe; J, *Diphyllobothrium osmeri ex* dog; K, *D. osmeri ex* dog; L, *Diphyllobothrium* sp. indet. *ex* man; M, *Diphyllobothrium dalliae ex* man; N, *D. dalliae ex* dog; O, *D. dalliae ex* man; P, *D. dalliae ex* arctic fox; Q, *D. dalliae ex* man; R, *Diphyllobothrium ursi ex* brown bear; S, *D. ursi ex* brown bear; T, *Diphyllobothrium alascense ex* dog; U, *D. alascense ex* dog; V, *D. alascense ex* dog; W, *D. alascense ex* dog; X, *Diphyllobothrium ?dendriticum ex* herring gull; Y, *D. ?dendriticum ex* herring gull, and Z, *D. ?dendriticum ex* glaucous-winged gull.

METHODS

Eggs of *Diphyllobothrium dalliae* for fresh water, *D. cordatum* for the marine environment, and *Diphyllobothrium* sp. indet. for brackish water were used. The larvae were either hatched or immersed (in the case of *D. dalliae*, since it will not normally hatch in saline situations) in solutions whose salinities ranged, in increments of 0.1 or 0.2 percent sodium chloride, from distilled water to one simulating normal sea water (3.5 percent). Following a 2-hour exposure to a particular solution, some larvae were killed with dilute formalin and measured.

RESULTS

Results are shown in figure 3. In distilled water, the greatest degree of swelling occurred in the larvae of *D. cordatum* which expanded on the average from 41 microns to 49 microns during a 2-hour exposure. At salinities of 2.8 percent and higher, these larvae retained a minimum diameter.

Larvae of the species adapted to brackish conditions presumably attained isotonicity at a salinity of 1.1 percent, since swelling was not evident, and showed little

change at 1.8 percent. *Pungitius pungitius,* the host for the plerocercoid stage of this cestode, lives in brackish ponds having salinities varying from 0.3 to 2.5 percent.

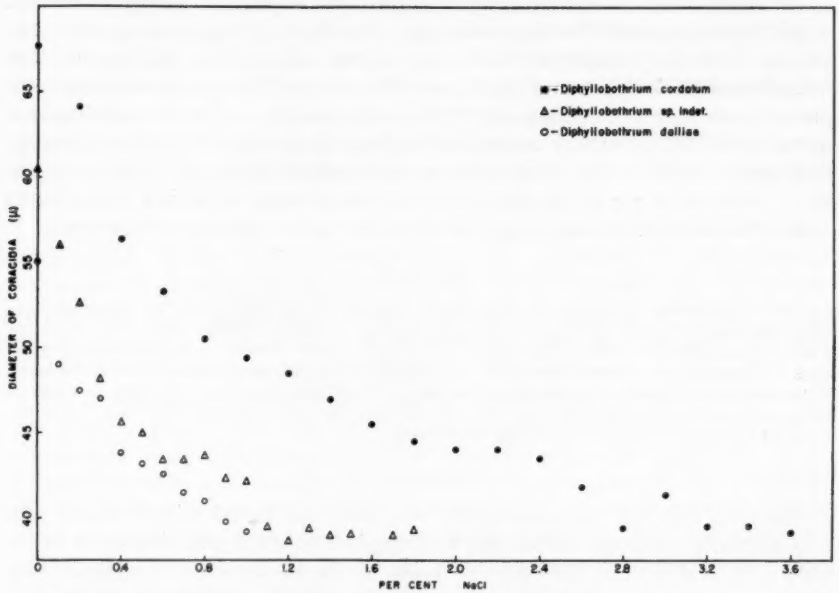

FIGURE 3. Reaction of coracidia of 3 species of *Diphyllobothrium* to differences in salinity. Each point on the curves represents measurements for 10 larvae exposed to given concentrations for 2 hours.

Data for the fresh-water species, *D. dalliae,* represent only empirical values. Minimal measurements do suggest, however, that the embryophore fluids may have a salinity of about 1 percent.

To establish the saline requirements of the other diphyllobothriid species, a regime was employed similar to that given above. In general, these species fell into one of three categories previously discussed. However, it was found that the larvae of *Schistocephalus solidus* could embryonate, hatch, and live in either brackish or fresh water; hence, this species is believed to be euryhaline. On the basis of these observations, the species are classified as follows:

Fresh water: *D. dalliae, D. ?dendriticum, D. ditremum, D. osmeri,* and *D. ursi.*

Euryhaline: *Schistocephalus solidus.*

Brackish: *Diphyllobothrium* sp. indet. (*ex Pungitius pungitius*).

Marine: *D. alascense, D. cordatum, D. lanceolatum, Diploganoporus balaenopterae,* and *Pyramicocephalus phocarum.*

Hydrostatic Pressure

Little consideration has been given the role of water pressure on embryonic development and hatching of diphyllobothriid eggs, other than the work of Vogel (1929), who found that the eggs of *Diphyllobothrium latum* would not hatch when subject to a pressure exerted by a column of water 15 cm high. In Alaska, where

diversified habitats exist, ranging from fresh through brackish, to marine, pressure could be a significant influence on the propagation of these cestodes.

If Vogel's observations are generally applicable to species of *Diphyllobothrium*, it is difficult to reconcile the high incidence of tapeworm infections among the fishes in some of the deeper lakes of Alaska, e.g., Karluk Lake, a deep oligotrophic basin in south-central Kodiak Island. Here, over 90 percent of the red salmon contain the plerocercoids of *D. ursi* (cf. Rausch, 1954), with which brown bears become infected by eating the fish when they return to the tributaries to spawn. The cestode eggs then, presumably, are deposited in the streams with the feces and washed into the lake. Since there are no shallow littoral areas, the eggs must find their way to depths of at least 24 feet as measured at the zone of confluence of the tributaries.

METHODS

To test whether eggs might hatch at depths greater than hitherto reported, the writer obtained specimens of *Diphyllobothrium ?dendriticum* from a glaucous-winged gull, while at Karluk Lake in late September, 1958. Eggs free of debris were placed in small, open, unstoppered glass vials and lowered into 1-quart plastic jars by nylon threads which were then secured to the necks of the open jars. Seven of these were attached at 5-foot intervals to a nylon line, one end of which was anchored in the lake at a depth of 35 feet; the other was buoyed 5 feet below the surface to prevent damage by ice during the winter.

RESULTS

On May 19, 1959, the samples were recovered and treated with formalin. An examination of the eggs showed that hatching had occurred sometime prior to recovery, as evidenced by the fact that many of the empty shells contained coiled trichomes of blue-green algae and frustules of diatoms. All eggs, irrespective of depth, demonstrated over a 90 percent hatch; the remaining eggs appeared infertile, since there was no evidence of embryonation.

Considering the vast difference in response to pressure shown by *D. latum* and *D. ?dendriticum* (difference in depth of 0.15 m and 10.7 m, respectively), it is quite likely that other members of the family hatch at depths intermediate to those cited or, perhaps, at even greater depths in the case of the marine cestodes.

DISCUSSION

It appears that the eggs of the species studied can be separated according to the environment of the last intermediate host (viz, fresh water, brackish, or marine). In those species whose larval development takes place in marine situations, the eggs usually have a width : length ratio greater than 1 : 1.45, while those developing in fresh water have a lower ratio. In the former, the surface of the shell is scrobiculate, with the size and arrangement of the pits differing according to species (pl. I, fig. 2–6). In species whose larvae develop in fresh water, the shells of the eggs are smooth and plain (pl. I, fig. 1). In addition, the shells of marine forms exceed 2 microns in thickness, while the shells of fresh water species only slightly exceed 1 micron in this dimension. In the former, the opercular sutures, viewed in optical section, are deeply notched; in the latter they are smooth.

According to available data, mean measurements of eggs are significant if only fertile ones are considered and if, for the same species of cestode, the final hosts are of the same class (i.e., either a mammal or a bird). Eggs are usually smaller from an avian host (e.g., *D. dalliae*, and *D. ursi*). Also, eggs of a species occurring in

pinnipeds are generally smaller than are those of the same species from terrestrial mammals (e.g., *D. cordatum*).

For the species of cestodes studied, the measurements of the embryonic hooks appear to have no taxonomic value. In some species, considerable variation in shape of hooks was noted in coracidia deriving from a single segment. In four species, *Diphyllobothrium lanceolatum, D. cordatum, D. ditremum,* and *Diplogonoporus balaenopterae,* the shape of the hooks appeared to be characteristic but in *D. ursi, D. alascense, D. osmeri, Pyramicocephalus phocarum, D. dalliae, D. ?dendriticum,* and *Schistocephalus solidus,* that feature varied so much as to have little diagnostic significance. The more typical forms are drawn in figure 1.

In the past, the possible taxonomic significance of plastin cells has received little consideration. Hussey (1950) reported that the coracidium of *Schistocephalus solidus* could be distinguished from three species of Diphyllobothrium by the greater average number of plastin cells; actual counts, however, were not given. Vogel (1929) noted that the coracidium of *D. latum* has from 8 to 12 such cells, and Kuhlow (1953) found an average of 10 cells in *D. dendriticum.*

For a given species of cestode, the writer found that there is a considerable range in numbers of plastin cells. In attempting to determine their significance, plastin cells were counted in 400 randomly selected coracidia of a known species of *Diphyllobothrium.* When the number of coracidia possessing a given number of plastin cells was plotted against the latter value, a normal distribution pattern was obtained with a high mean and low extreme values. Comparable data were obtained with the same species, but from different hosts also with samples of coracidia ranging from 100 to 400 per cestode.

Similar observations were made on eight additional species of cestodes. As shown by Dice-Laarus graphs (fig. 2) and numerical analysis (under "Results"), this method appears to be reliable as a means of distinguishing certain species of diphyllobothriid cestodes. In each case, the number of plastin cells is characteristic of the species and distinguishes it from others, except for *Diphyllobothrium* sp. indet. and *D. dalliae,* which showed comparable counts. Plerocercoids of both species, however, are known to occur in *Pungitius pungitius* and additional work is needed to clarify taxonomic differences.

It is evident that range and mean values are similar for the same species from different hosts. The mean was found to vary between 0.12 and 0.79, but usually this was only about 0.3. The only exception was in *Schistocephalus solidus,* where the difference in mean between specimens from a red-necked grebe and a dog was 1.02; however, with that species from dogs, taken at two localities 75 miles apart, the difference in mean was only 0.02. It appears that the host may affect the mean number of plastin cells, but additional work is required to confirm this.

Vogel (1929) found that the eggs of *Diphyllobothrium latum* would not hatch unless they were exposed to light. The writer obtained spontaneous hatching of large numbers of coracidia by holding embryonated eggs in total darkness for a day or so beyond the predetermined incubation period and then exposing them to light. It was observed, however, that the eggs of certain species would hatch in darkness at 20 C; in *D. dalliae,* over 60 percent hatched after the prescribed period, while in *D. ursi,* 20 percent hatched after 31 days. With *Diphyllobothrium* sp. indet., nearly 100 percent of the coracidia emerged in the dark after the normal time of incubation.

The tundra pools in which the eggs of *D. dalliae* and *Diphyllobothrium* sp. indet.

are disseminated have water containing a fair concentration of humic colloids. This condition lends a dark color to the water, often giving it a coffee-like appearance. Much of the incident light, therefore, may be absorbed before it reaches the bottom where the cestode eggs come to rest. Secchi disc measurements in such water are usually less than 0.5 meter.

In the case of *D. ursi* at Karluk Lake, depth of water may prevent light reaching the eggs, since they are usually at depths of over 24 feet. Also, the bottom of this lake is covered with a diatomaceous ooze which may envelope the eggs and hence further obstruct the passage of light.

In his studies on *Diphyllobothrium latum,* Vergeer (1936, p. 721) states: "There seems to be a definite correlation between the time required for the hatching of the eggs and the length of life of the coracidia, both being conditioned by the temperature of the water." The writer found little evidence to support this hypothesis. For example, *D. ?dendriticum* requires from 6 to 8 days to hatch at 20 C, but dies within 18 hours when kept at this temperature; at 2 C it lived for 4 days. *D. cordatum,* however, requires only 5 to 6 days' incubation at 20 C before emerging, but survived at that temperature for 2 days; at 2 C most of the larvae were still swimming on the 104th day. Nine days were needed before coracidia of *D. ditremum* hatched, yet they lived but 10 hours at 20 C, and 3 days at 2 C. The response of *Schistocephalus solidus* to temperature showed an inverse relationship by comparison with the other species studied and also with *D. latum;* in fresh water, *S. solidus* required from 14 to 16 days to hatch when held at 20 C, at which the coracidia lived 8 days after hatching; they lived only 2 days at 2 C.

Vergeer stated further that cold temperatures as maintained by modern electric refrigerators (temperature not stated) almost completely arrested development of the embryo of *D. latum,* and that according to La Rue (cited in Vergeer) eggs could be kept viable for three and one-half years provided they are held at sufficiently low temperatures (again, temperature not stated). When Alaskan cestode eggs were stored at 2 C, it was found that those of *D. ursi* and *D. dalliae* hatched at that temperature in 4 to 5 months in the absence of light. Eggs of *D. ?dendriticum* were found fully embryonated and active but required light to hatch after being stored for 5 months at 2 C. Eggs of *Schistocephalus solidus* kept at this temperature for a short period would not thereafter embryonate at room temperature. These observations and failure of the coracidia of this species to survive as long in cold water as in more temperate situations, suggests that *Schistocephalus solidus* is restricted to warm-water situations; however, each species must be considered separately, and generalization regarding hatching is not justified.

SUMMARY

Eggs and coracidia of 11 species of diphyllobothriid cestodes were examined for characteristics of possible taxonomic value. On the basis of egg morphology, species could be separated according to the environment of their last intermediate host, i.e., marine or fresh water. Embryonic hook measurements were not found to be valid as taxonomic characters. Hook form was fairly diagnostic for *Diphyllobothrium lanceolatum, D. cordatum, D. ditremum,* and *Diplogonoporus balaenopterae;* while in *D. ursi, D. alascense, D. osmeri, D. dalliae, D. ?dendriticum, Pyramicocephalus phocarum,* and *Schistocephalus solidus* the hook structures were too variable to be of diagnostic value.

Plastin cell counts appear to have considerable taxonomic value. Statistical consideration shows their mean and range to be in close agreement for a given species irrespective of the host in which it occurs.

Observations were made on the living coracidia of most species and consideration given the rate and pattern of swimming, and the responses to variation in temperatures and degree of salinity.

The effects of temperature, pressure, and light on the hatching of eggs are discussed.

ACKNOWLEDGMENTS

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EXPLANATION OF PLATE I

FIGURE 1. Egg of *Diphyllobothrium dalliae*, showing smooth and undifferentiated texture of shell.

FIGURE 2. Egg of *Diphyllobothrium alascense*, showing scrobiculate texture of shell.

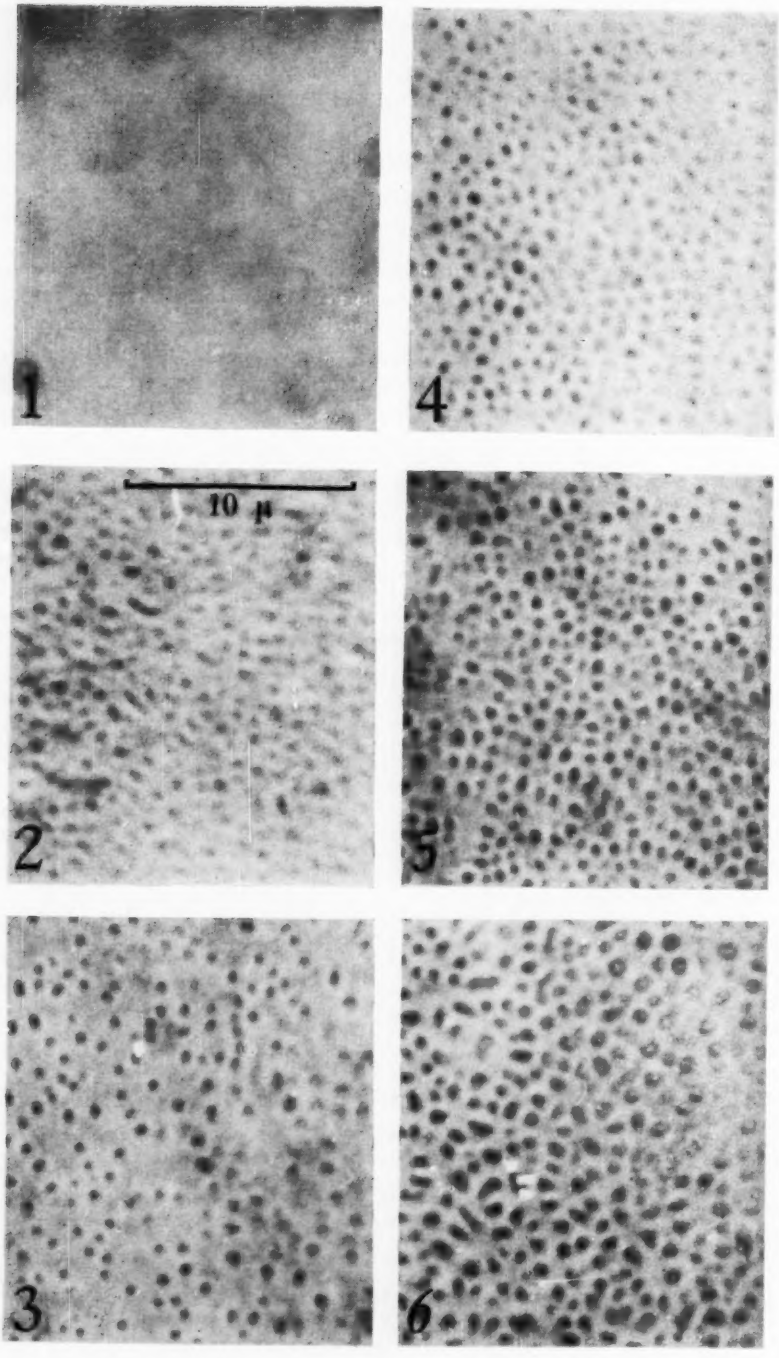
FIGURE 3. Egg of *Pyramicocephalus phocarum*, showing scrobiculate texture of shell.

FIGURE 4. Egg of *Diphyllobothrium lanceolatum*, showing scrobiculate texture of shell.

FIGURE 5. Egg of *Diphyllobothrium cordatum*, showing scrobiculate texture of shell.

FIGURE 6. Egg of *Diplogonoporus balaenopterae*, showing scrobiculate texture of shell (all photographs at 1200× and enlarged).

PLATE I



STUDIES IN CYSTICERCOID HISTOLOGY. IV. OBSERVATIONS ON HISTOGENESIS IN THE CYSTICERCOID OF *HYMENOLEPIS DIMINUTA* (CESTODA: CYCLOPHYLLIDEA)*

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The growth of *Hymenolepis diminuta* in the intermediate host has been described by Voge and Heyneman (1957) who studied the succession of gross morphological changes in normal development. The normal histologic structure of fully developed *H. diminuta* cysticercoids has been described by Voge (1960). This paper presents the major histologic changes which occur during normal development of this species in the intermediate host. It should serve as a basis in the recognition of abnormal development produced experimentally since abnormalities are not always evident upon observation of gross structure (Voge, 1959a, b). In addition, the description of the fully developed cysticercoid (Voge, 1960) will be amended and enlarged, and the origin of at least some of the tissues discussed.

MATERIALS AND METHODS

Materials and methods were as described by Voge and Heyneman (1957). Designation of developmental stages also follows the system of these authors. All studies were made on organisms developed in *Tribolium confusum* kept at 30 C. Observations, which were begun at stage 2 of development, are based on paraffin-embedded material fixed in Zenker's fixative and sectioned at 7 microns. Stains used were Heidenhain's iron-hematoxylin, Meyer's hemalum (Lillie, 1954), Himes' triple stain (Himes and Moriber, 1956), Gomori's trichrome (Voge, 1960), and Mallory's aniline blue collagen stain (Gridley, 1953). The last-named was particularly useful in tracing the development of all fibrous tissues.

DESCRIPTION

As shown previously (Voge and Heyneman, 1957), the early stage 2 at 3 days development is a spherical structure which contains an eccentric cavity (plate I, fig. 1). The majority of the nuclei are concentrated in the area which will become the anterior end of the cysticercoid and are of two sizes. The larger nuclei are more numerous near the edge of the cavity while the smaller ones are present in larger numbers near the periphery. Fine strands appear to connect some of the larger nuclei and similar strands also protrude into the cavity. Very few definite cells are present. Some of these are elongate or spindle-shaped and are situated near the edge of the cavity. Just beneath the external membrane on the surface of the sphere are fine, parallel striations (plate I, fig. 1) observed only with high magnification. All tissues stain pink to red with Mallory's stain.

As the sphere grows it elongates and in 3.5 to 4 days becomes a pear-shaped body (plate I, fig. 2) in which the wider end contains a relatively dense accumulation of small nuclei, the area of the future scolex. Posterior to this there are fine strands of tissue oriented transversely and bordering the cavity. Associated with these strands are large nuclei. Smaller amounts of similar tissue may be observed in the middle of

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the cavity. The elongate cells observed in the spherical stage are also present. In some sections, the cavity is seen to contain a very fine acellular network (plate I, fig. 2) which may be an artifact. It should be noted that it is very difficult to ascertain the relationship between the various nuclei and the cytoplasmic components of these larval tissues. It appears as if most of the nuclei were lying within an irregular meshwork of cytoplasm, with a few definite cells along the periphery of the organism. The chromatin in all nuclei is in the form of several granules. The nuclei are now of three sizes (plate II, fig. 8). The largest nuclei are primarily situated in the transverse strands within the cavity. Those intermediate in size are seen near the periphery of the organism, either free of cytoplasm or within some of the elongate cells. The smallest nuclei are most numerous and are present throughout, particularly in the future scolex and tail regions. With Himes' triple stain the external membrane and all extranuclear cytoplasm stain an intense reddish-purple. The color is most intense in the center of the future scolex and in the middle portion of the body.

Further growth and elongation of the organism results in the tripartite body division designated previously as stage 3. Figure 3 of plate I illustrates the extent of tissue differentiation which has occurred at this stage at 5 days of development. Outlines of the scolex and suckers are well established. The mid-portion of the body contains a large cavity. Within the cavity, the strands of tissue which were transversely oriented in the pear-shaped stage, have increased in extent and form a cup-shaped structure or plug. Anterior to this plug a highly vacuolated area may be seen to extend almost to the level of the suckers. In some sections, this area appears as a canal or tube containing nothing but a few strands of tissue like that forming the plug. Posterior to the plug, within the cavity are scattered small, spindle-shaped cells some of which appear to be in contact with delicate fibers. These cells contain nuclei of the smallest size. The tail usually contains one or two large cavities free of cells. The tissue in the periphery of the tail is less dense than elsewhere.

A very distinct alignment of cells has occurred in the mid-body of the organism. The cells observed in earlier development have increased in number, become elongated, and attach to the periphery of the organism by means of narrow cytoplasmic processes (plate I, fig. 3). Cells of this type are never observed in the scolex or in the tail. They contain nuclei of the intermediate size. In addition to these peripheral cells, there are small nuclei, apparently free of cytoplasm, situated between the cells and close to the periphery of the organism. Eventually, further elongation occurs in the cells of the cavity. Some of these cells are oriented in a definite longitudinal direction with cytoplasmic processes several times the length of the original cell (plate II, fig. 5). Others may be oriented transversely and develop more than two cytoplasmic processes so that they eventually appear roughly star-shaped with many fine processes of varying length. The relatively delicate fibers shown in plate I, figure 3 become more pronounced and more numerous. Well before the time of scolex withdrawal, these fibers become organized into groups or delicate sheets of variable thickness and extent, oriented toward the free ends of the elongate peripheral cells. At the time of scolex withdrawal, contact between the peripheral cells and the fibers has already been established so that the appearance of a section through this area before scolex withdrawal is very similar to that shown in plate I, figure 4.

Sections stained with Himes' triple stain indicate increased polysaccharide con-

tent in the scolex. The tissue strands in the cysticercoid cavity, the tail and the external membrane stain a deep purple. The cytoplasm of the peripheral cells stains a bright yellow. With Mallory's stain, all tissues stain red except for the fibrous plug which now has a reddish-purple cast.

The next step in development is withdrawal which occurs at 5.5 to 6 days and, when accomplished, represents stage 4. Plate I, figure 4 illustrates a longitudinal section through an organism at 6 days development, containing the withdrawn scolex. This stage differs from the preceding 5-day stage in several ways. Scolex withdrawal into the cavity has resulted in a change in position of the two types of tissues seen in the cavity before withdrawal. The anterior plug has been pushed further inside the cavity by the receding scolex and has come to lie posterior and lateral to it, subsequently completely surrounding the scolex and "neck" tissue and lining the whole cavity. The fibroid tissue within the cavity posterior and lateral to the plug has also been compressed and pushed back so that it now forms a second distinct layer, the future fibrous layer of the fully developed organism. The limits of the cysticercoid cavity are now sharply defined. The lining tissue within still contains the relatively large, spherical or ovoid nuclei with well defined nucleoli. By comparison, the fibroid tissue outside the cavity contains the small nuclei, most of which are still spherical although a few have become compressed and elongate. The empty spaces, alternating with the fine strands of tissue in contact with the peripheral cells (plate I, fig. 4) are observed in sections of all organisms at this stage of development. An additional type of structure may now be observed within the fibrous tissue outside the cavity. It consists of a very long and relatively thick cord, bending and sometimes branching in irregular fashion and containing several vesicle-like enlargements (plate II, fig. 7). These thick cords stain a deep red with Mallory's stain while the enlarged portions do not stain. The course of any one of these structures cannot be followed completely in sectioned material but they may be seen to extend from the edge of the cavity to the peripheral cells with several irregular folds throughout their course. Some of them terminate in a bulb-like arrangement near the periphery of the organism (plate II, fig. 6). The development of these structures could not be followed.

Changes in the peripheral cells include a gradual withdrawal from the periphery which is accomplished by further elongation of the distal cytoplasmic processes. In some of the cells the connection with the periphery of the organism is no longer apparent. The peripheral cells will constitute the intermediate cell layer (Voge, 1960) of the fully grown cysticercoid. The tissues of the scolex have become denser and the outline of the suckers more sharply defined. The nuclei within the suckers are aligned in a regular manner (plate I, fig. 4). Staining reactions have changed from those observed before scolex withdrawal in that a distinct color separation now exists between the lining tissue of the cavity and the fibrous tissue outside the cavity. With Mallory's stain, all fibrous tissues outside the cavity, including the fibrous extensions or processes directed toward the peripheral cells, appear pale purplish-blue. All other tissues are varying shades of red. As development proceeds, these fibrous tissues lose their purplish cast and become an intense blue. From this point on, additional growth occurs primarily in the fibrous tissues. The fibrous layer becomes more compact and the fibrous processes more numerous. At 7 days, these processes extend to the periphery of the organism where they bend and extend further so that their terminal portions become situated parallel with and just beneath the surface of

the organism. Plate II, figure 1 shows the position of the fibrous processes in relation to the peripheral cells. It appears as if these cells direct the growth of the fibers which surround them on both sides; the fibers follow the cytoplasmic extensions of the cells toward the periphery. The cytoplasmic extensions of the peripheral cells have also elongated further and progressed in a longitudinal direction parallel with the surface. The spatial arrangement of fibrous processes and the cytoplasmic processes is shown in the cross-section in plate II, figure 1. Free nuclei of two sizes lie close to the surface in the spaces between the peripheral cells. The small nuclei in the fibrous layer have become elongate and somewhat pycnotic. The nuclei of the lining layer of the cavity, however, have retained their shape and are apparently unchanged. The scolex has a clearly demarcated, thick cuticle which stains red with Mallory's stain and yellow with Himes' stain.

Stage 5, reached at 8 days development, shows staining reactions similar to those observed at 7 days. Structural changes include larger numbers and further growth of the fibrous processes. The distal portions of these processes appear as fine hairs and represent the hairy processes described previously for the fully developed organism (Voge, 1960). Plate II, figure 2 shows the relationship of the various tissue layers. Comparison with plate II, figure 1 shows an enlargement of the peripheral cells as well as a considerable thickening of the area near the periphery of the organism. This is in part due to growth of the hairy processes with concomitant increase of fibrous portions lying parallel to the surface. In addition to the tissues already described, a new layer can now be observed under high magnification. It is a circular layer of very fine, transversely oriented fibers, situated at the point of outgrowth of the hairy processes, and external to that portion of the fibrous processes which lies parallel with the surface.

The development of this circular layer, which in its position is similar to that described for *H. nana* (Voge and Heyneman, 1960) could not be followed but it is possible that the fine striations observed in stage 2 and later stages represent the initial steps of development. It is of interest that beneath this circular layer one may find star-shaped cells (plate II, fig. 4) like those observed in a similar location in *H. nana*. The small nuclei scattered among the peripheral cells are the nuclei of these star-shaped cells, the cytoplasm of which lies parallel to the surface and can be observed only in very superficial sections. It is of interest that the hairy processes grow through the fine, circular fibrous layer. Whether or not the circular fibers also contribute to the hairy processes could not be established. There is no doubt, however, that the orientation of the hairy processes is a radial one, because their position is similar in longitudinal and in cross-sections. The distal ends of the hairs come in contact with the surface layer of the organism just beneath the external membrane. The surface, when viewed from the outside, is a delicate network composed of small, roughly hexagonal units which stain red with Mallory's stain and are visible only under high magnification ($\times 900$ or higher).

To summarize briefly, the sequence of structures from the outside toward the center of the organism is as follows: (1) external membrane, (2) surface network, (3) hairy processes, (4) fine circular fibers, (5) prominent, primarily longitudinal, folded extensions of the fibrous processes, (6) star-shaped cells, (7) main body of the peripheral cells, (8) fibrous layer, (9) lining of the cavity, and (10) tissue surrounding the scolex, with the scolex proper. The cysticercoid is now fully developed in that any additional changes are either retrogressive or represent further

elaboration of already existing tissues or layers.

Of interest in this regard are observations on the organization of 5.5 months old cysticercoids. Comparison of plate II, figure 3 which shows the tissues of such a cysticercoid, with young cysticercoids (plate II, fig. 2) shows the pronounced changes which have occurred with age. The large cells originally near the periphery of the cysticercoid have deteriorated so that in most instances only their nuclei are clearly visible. The area occupied by them has decreased in width. Because of the extensive development of the fibrous processes and particularly the hairs, these cells which originally occupied a peripheral position are now truly intermediate between the fibrous layer and the thick bundles of fibers beneath the thin circular layer (plate II, fig. 3). In some specimens, the hairy processes are much longer than shown.

DISCUSSION AND SUMMARY

While the observations reported here have established some of the details of histologic differentiation during development of *Hymenolepis diminuta* cysticercoids, the precise manner in which this differentiation occurs is unknown. One of the most interesting problems is that of the origin of the fibrous tissues. It is not clear whether individual fibers are formed as a result of dedifferentiation of cytoplasm of the various spindle-shaped and star-shaped cells, or whether they are produced by secretion from these cells. There is no doubt that the spindle-shaped cells in the cavity are in some way associated with fiber formation.

Similarly, the star-shaped cells, which have already been described in *Hymenolepis nana* (Voge and Heyneman, 1960), are found in close association with superficial fibrous layers. The function of the large peripheral cells is unknown except that they in some way determine the direction of growth of the fibrous processes from the fibrous layer.

In relation to excretory processes, one can speculate about the function of the long cord-like structures represented in plate II, figures 6 and 7. While flame cells have been observed in the scolex in living material, none was seen in tissues external to the cavity. The presence of small vesicular enlargements in the long structures seen in tissues outside the cavity suggests the possibility that they may perhaps function in excretion. The detailed morphology of these, as well as other tissue components, requires further study.

In the description of the histology of the fully grown cysticercoid several details were omitted (Voge, 1960). One of these is the layer of the fine circular fibers, another the presence of the star-shaped cells. The structures previously called "cells of the peripheral layer" are in reality thick bundles of fibers enclosing cytoplasmic extensions from the large cells which during development were close to the periphery. As already stated, these cells assume an intermediate position in the fully developed cysticercoid. The relatively thick extensions of the fibrous processes which lie beneath the circular fibers were not described originally.

In view of the complexity of histologic organization of the cysticercoid of *Hymenolepis diminuta*, many more observations are needed to extend our knowledge of this organism. New methods of study, including electron microscopy, should be used to demonstrate the fine structure of the fibers, of the external membrane and of the surface layers. The formation of the cuticle of the scolex and the muscles of the suckers also require further investigation. Culture in vitro, when it becomes possi-

ble, should contribute significantly to our understanding of differentiation of the cysticercoid.

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Explanation of Plates

All figures drawn with the aid of a camera lucida. Abbreviations: ae, anterior end; an, acellular network; c, cavity; cf, circular fibers; ec, elongate cells; em, external membrane; f, fibers; fl, fibrous layer; fp, fibrous processes; hp, hairy processes; lc, lining of cavity; p, plug; pc, peripheral cells; sc, spindle-shaped cells; ts, transverse strands.

Plate I

FIGURE 1. Section of spherical stage 2, developmental period 3 days, showing eccentric cavity, elongate cells, and fine parallel striations on the surface of the organism; the relatively prominent mass of small nuclei represents the anterior end.

FIGURE 2. Section of pear-shaped stage 2, developmental period 4 days, showing the presence of tissue strands with large nuclei anteriorly and the fine acellular network within the cavity.

FIGURE 3. Section of stage 3, developmental period 5 days, showing tripartite body division, alignment of peripheral cells, the presence of a plug which has developed from the tissue strands shown in figure 2, spindle-shaped cells, and fibers within the cavity, as well as outlines of suckers in the scolex.

FIGURE 4. Section of stage 4, developmental period 6 days, showing the withdrawn scolex, and the relationship of the lining of the cavity formed from the plug to the adjacent fibrous layer; the extensions or fibrous processes from the fibrous layer are in contact with the peripheral cells.

Plate II

FIGURE 1. Section of tissues outside cysticercoid cavity, developmental period 7 days; the peripheral cells are in contact with the surface by means of their cytoplasmic extensions; the fibrous processes from the fibrous layer are situated close to the margins of the cells and extend to the periphery of the organism on either side of the cytoplasmic extensions.

FIGURE 2. Section as in figure 1, developmental period 8 days; note size increase of peripheral cells, extensive development of fibrous processes which bend at right angles and are situated parallel with and beneath surface as are cytoplasmic extensions of peripheral cells (the latter not shown in entirety); growth of the hairy processes has resulted in increase of space between the distal portion of fibrous processes and the surface of the cysticercoid beneath the external membrane.

FIGURE 3. Section as in figures 1 and 2, age 5.5 months; note marked increase in fibrous processes, grouped into thick bundles, especially just beneath the delicate circular fibers; parts of cytoplasmic extensions of the former peripheral cells are evident within each bundle and are shown here as black dots; because of extensive growth of hairy processes, as well as other fibers, the cells which were initially peripheral are now in a position intermediate between the fibrous layer and the other fibrous structures; cells have degenerated and only pycnotic nuclei remain.

FIGURE 4. Star-shaped cells seen beneath parts of fibrous processes parallel with the surface.

FIGURE 5. Elongate cells from cavity seen at 5 days of development.

FIGURES 6, 7. Structures observed in fibrous layer at 7 days development, showing clear, vesicular portions, and branching; rarely, branches appear to be in contact with nucleus (fig. 7).

FIGURE 8. Three types of nuclei from organism at 5 days development; the smallest seen primarily in the scolex and tail, the largest in the plug or lining of the cysticercoid cavity, and the intermediate throughout the tissues external to cavity.

RESEARCH NOTE

A METHOD OF HANDLING SMALL PARASITES DURING STAINING PROCEDURES.

Small helminths, such as the microphallids and gymnophallines which may be as small as 100 microns by 50 microns, require special care to prevent loss and damage during hydration, staining and dehydration. Heil (1933, Stain Tech. **8**: 149–151) used silk screening cloth on histological rings for bulk staining of *Paramecium*. Histological rings are difficult to obtain and are opaque black. Stained helminths are not apparent against a black background if, by chance, they stick to the side of the chamber. To overcome these difficulties, small (15/16 by 15/16 by 3/4 inch) clear plastic boxes (available from Braun Corp.) were used. The bottom was removed from the box with a red-hot fine tipped scalpel blade and the box was placed, bottom down, in a one-eighth-inch layer of xylene until the immersed edge was soft. Softening usually required about 5 minutes. The plastic box was then placed, top down, on a glass plate under a dissecting microscope. A one-inch square of 200 mesh silk bolting cloth was placed on the bottom of the box and pressed into the soft plastic with a dissecting needle. The xylene was evaporated from the plastic in an open room or in a 60 C oven. When the plastic was hard the excess silk was trimmed with a sharp scalpel.

The boxes containing the worms may be transferred through hydration solutions, left in stain the required time, then dehydrated to about 80 percent alcohol. An absorptive tissue was used to remove excess fluid when the boxes were being transferred from one solution to the next. Destaining in acid 70 percent alcohol does not harm the boxes, nor does cold AFA fixing solution. Solutions were kept in deep petri dishes, which held seven boxes each.

This clear plastic box with a silk bolting cloth bottom has a number of advantages. (1) The worms do not have to be handled directly from fixation until dehydration in 80 percent alcohol. (2) Loss of small forms is kept to a minimum. (3) The clear plastic allows evaluation of the state of the worms at all stages. (4) The boxes are easy to handle. The main disadvantage is that the boxes must be kept away from organic solvents and therefore the worms must be removed from the boxes for clearing. The silk stains but worms are still visible and destaining removes this stain.—HOMER T. RUSSELL, JR., *Department of Zoology, University of California at Los Angeles.* Present address: *Department of Biology, University of California at Santa Barbara, Goleta, Calif.*

PLATE I

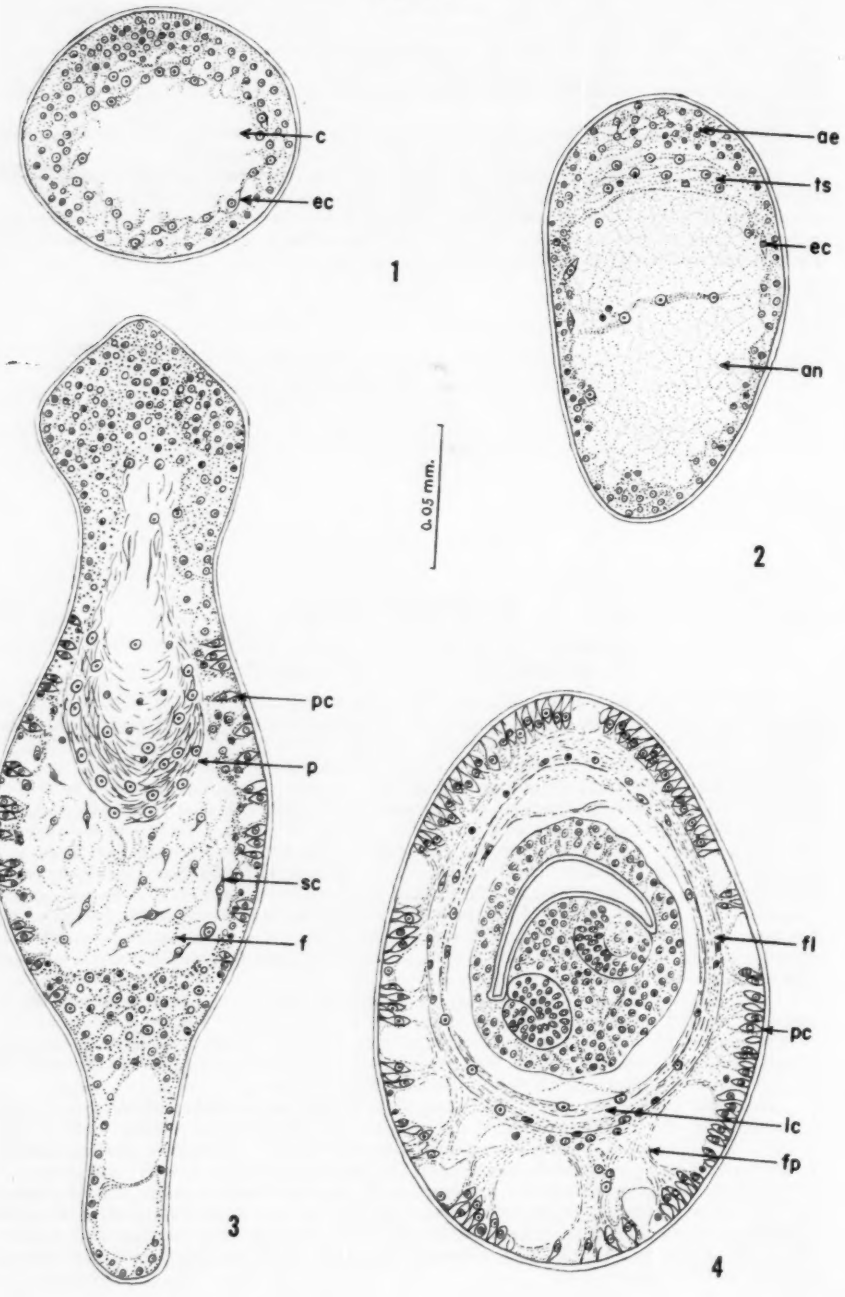
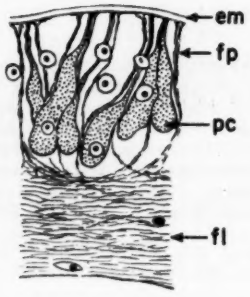


PLATE II



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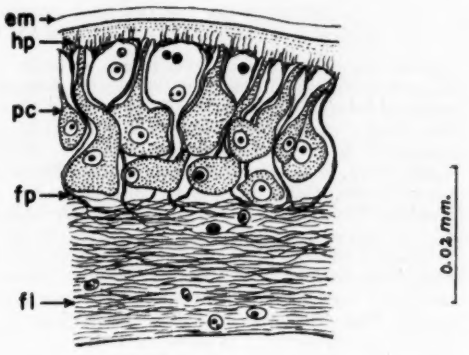

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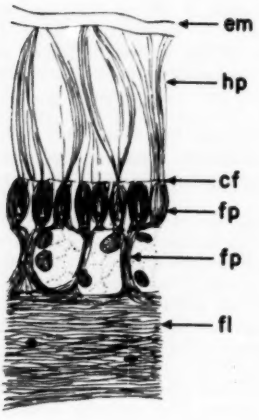

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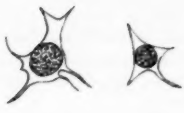

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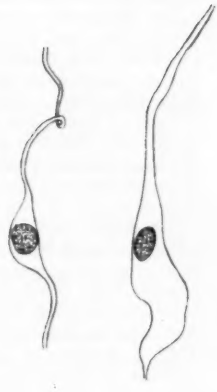

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RESEARCH NOTE

A RECORD OF *PHARURUS OSERKAIAE* (SKRJABIN, 1942) IN AN ALASKAN WHALE.

Studies on the parasites of the beluga or white whale (*Delphinapterus leucas*) have been comparatively limited because of difficulty in obtaining the hosts. Seven genera comprising 10 species of helminths are listed by Deliamure and Kleinenberg (1958, *Biul. Moskov. Obsh. Ispyt. Prir., Otol. Biol.*, n. s. **63:** 25–32): (TREMATODA) *Odhneriella seymouri* (Price, 1932) and *Leucasiella mironovi* Krotov and Deliamure, 1952; (NEMATODA) *Anisakis kukenthalii* (Cobb, 1889), *A. simplex* (Rudolphi, 1809), *Otophocaenurus oserskoi* Skrjabin, 1942, *Stenurus minor* (Kuhn, 1829), *S. arctomarinus* Deliamure and Kleinenberg, 1958, *S. pallasii* (van Beneden, 1870), and *Crassicauda giliakiana* Skrjabin and Andreeva, 1934; (ACANTHOCEPHALA) *Corynosoma strumosum* (Rudolphi, 1802).

Several hundred pseudaliid nematodes were collected from head sinuses of a beluga whale, taken from the sound off Kotzebue, Alaska. Apparently the helminths are not uncommon in occurrence since Eskimo natives of the village previously have encountered them while removing flesh from whale skulls. Morphologically the parasites fit the redescription of *Otophocaenurus oserskoi* as given by Deliamure (1955, *Helminths of Marine Mammals, with Emphasis on their Ecology and Phylogeny*. Moscow. *In Russian.*) Skrjabin recovered his material from the auditory apparatus of a cetacean from Sakhalin Island. Except for the original report, the writers have not found any additional occurrence records in the literature of this or of any other species of the genus. References to *Otophocaenurus*, however, were made by Dougherty (1943, Proc. Helm. Soc. Wash. **10:** 16–22; 1949, Parasitology **39:** 222–230), Deliamure (*loc. cit.*), and Deliamure and Kleinenberg (*loc. cit.*). In his latter paper, Dougherty offered a historical summary of the classification of the Metastrongylidae Leiper, 1909, and discussed his opinion of the origin and radiation of genera comprising the subfamily Pseudaliinae Railliet and Henry, 1909. On the basis of his studies, he placed the species *oserskoi* in the genus *Pharurus* Leuckart, 1958, and designated it *P. oserskaiaae* (Skrjabin, 1942). He further reaffirmed an earlier (1943) recommendation that the genus *Torynurus* Baylis and Daubney, 1925, is likewise a synonym of *Pharurus*. A further revision in the classification of the family Metastrongylidae was presented by Dougherty (1951, Parasitology **41:** 91–96). Based upon Article 14 of the International Rules of Zoological Nomenclature and certain decisions of the XIII International Congress of Zoology, *oserkaiaae* was emmended by him to *P. oserkaiae*.

Through the courtesy of Dr. Allen McIntosh of the Animal Parasite Laboratory, Beltsville, Maryland, the writers were permitted to examine several pseudaliin genera: *Torynurus*, from *Monodon monoceros; Pseudalius*, from *Phocoena phocoena; Sternurus*, from *Globiocephalus brachypterus; Otophocaenurus*, from *D. leucas*. The last was recovered from an Alaskan host, but, apparently, no record of it has appeared in literature. It was pointed out by McIntosh (personal communication) that the Parasite Laboratory regards *Pharurus alatus* (Leuckart, 1848) a synonym of *Torynurus alatus* (Leuckart, 1848) Deliamure, 1955. This opinion is in contrast to the present one of Dougherty (personal communication), as well as his earlier contentions (1943; 1949) that the two names are synonyms of each other, *P. alatus* having priority. From detailed personal comparative studies of the genera cited and the Kotzebue material, and with diagrams of these groups in the literature, it seems highly probable that *Otophocaenurus*, *Torynurus* and *Pharurus* are synonyms. *Otophocaenurus* appeared to differ primarily from the other two genera in the absence of a gubernaculum. On the other hand, the significant feature in *Pharurus* is the absence of the pre-bursal sucker, which is present in the other two groups. These singular differences do not seem sufficient to justify the existence of separate genera. Tentatively, the writers therefore concur with Dougherty in his synonymies. The nomenclature of Yorke and Maplestone (1926, *The Nematode Parasites of Vertebrates*, Churchill, London.) and Deliamure (*ibid.*) which assumes *Pharurus* to be a synonym of *Sternurus* Dujardin, 1845, apparently is incorrect because of the difference in spicular morphology in the two groups. Until additional specimens become available for making comparative studies, the alternative is to allow these two groups to remain generically distinct.

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THE EFFECT OF CECAL COCCIDIOSIS INFECTIONS AND EXPERIMENTAL HEMORRHAGE UPON ADRENAL ASCORBIC ACID LEVELS IN THE CHICKEN*

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The role of the adrenals in resistance to parasitic infection in birds has been given little attention. This has been due in part to an incomplete understanding of avian adrenal physiology, and to a lack of appropriate methods for measuring changes in adrenal function.

Many studies of mammalian species, and of the rat in particular, have employed the depletion of adrenal ascorbic acid as an indicator of response to stress (Long, 1947). Under certain conditions adrenal ascorbic acid depletion has been shown to be closely correlated with adrenocorticotropic hormone levels (Sayers, Sayers and Woodbury, 1948). The mechanism of ascorbic acid depletion and its relationship to the biosynthesis and secretion of adrenal cortical hormones, however, remains to be demonstrated.

Efforts to demonstrate stress or ACTH induced adrenal ascorbic acid depletion in birds have given negative results in most cases although other evidences of adrenal stimulation such as adrenal enlargement and cholesterol depletion have been observed (Jailer and Boas, 1950; Howard and Constable, 1958; Elton et al, 1959). Similarly, Josephson et al, 1949) found no change in the adrenal ascorbic acid content of chickens infected with *Plasmodium gallinaceum* although adrenal enlargement took place. A recent exception to these negative results is the report of Perek and Eckstein (1959) who observed adrenal ascorbic acid depletion after administration of ACTH to laying hens. This paper reports some preliminary observations on changes in adrenal ascorbic acid which take place in chickens infected with the protozoan parasite *Eimeria tenella*. Since hemorrhage is an important part of the syndrome caused by this parasite, data on the effect of experimental hemorrhage are also presented.

MATERIALS AND METHODS

The effect of coccidiosis infections upon adrenal (reduced) ascorbic acid was determined using the indophenol method of Carruthers (1942) in a series of Barred Rock × Rhode Island Red pullets which ranged in age from 6 to 12 weeks. The method of Roe and Kuether (1943) was employed to determine the adrenal total ascorbic acid (reduced ascorbic acid plus dehydroascorbic acid) in a series of White Rock cockerels which ranged in age from 5 to 10 weeks.

Coccidiosis infections were obtained by oral administration of washed, sporulated pure line cultures of *E. tenella* oocysts at a rate of approximately 100,000 oocysts per kg of body weight. All birds were fasted for 12 hours before administration of the oocysts in order to increase the uniformity and severity of the infections (Edgar and Herrick, 1944). This dosage produced severe symptoms of cecal coccidiosis with profuse hemorrhage from the intestinal ceca and marked loss of body weight.

White Rock cockerels 8 to 14 weeks of age were used in the experiments upon the effect of

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artificial hemorrhage. Blood was removed by heart puncture. One group of birds received repeated bleedings in which 10 ml of blood was drawn from each bird at 2-hour intervals over a 12-hour period. After an additional 12 hours, the birds were killed and the adrenals were quickly removed and analyzed for total ascorbic acid (Roe and Kuether, 1943). A second group of birds received single massive bleedings which ranged in quantity from 30 to 60 ml. In this series an attempt was made to remove as much blood as possible without causing the death of the bird. The birds were sacrificed 3, 6, and 12 hours later, and total ascorbic acid analyses were done as for the previous group. Determinations were carried out simultaneously on untreated controls and on birds which had been subjected to repeated heart punctures without removal of blood except for very small quantities used for hematocrit determinations (Natt and Herrick, 1955).

The birds were maintained in wire batteries and were kept free of infection until they were needed for experimental purposes. The Barred Rock × Rhode Island Red pullets were fed a ration which consisted of ground corn (500 lbs), wheat bran (225 lbs), soybean meal (75 lbs), wheat middlings (225 lbs), dried milk (75 lbs), alfalfa leaf meal (75 lbs), meat scraps (150 lbs), grit (27 lbs), and cod oil (3 lbs). All other birds were maintained on a commercial crumbelized grower ration (Master Mix 119).

RESULTS

Coccidiosis Infections: The adrenal ascorbic acid content of pullets with cecal coccidiosis showed a marked change during the course of infection (table I).

TABLE I. *The effect of cecal coccidiosis upon the adrenal ascorbic acid of Barred Rock × Rhode Island Red pullets.*

Day of infection	Body weight (gm)		Adrenal weight (mg)	Adrenal ascorbic acid (mg percent)	No. of birds
	Pre-infection	At sacrifice			
0		1446	94.4 ± 5.2*	121 ± 0.4*	24
3	736	820	74.3 ± 4.0	109 ± 3.1***	12
4	979	1083	77.7 ± 6.0	117 ± 1.1**	15
5	1153	1118	105.0 ± 7.3	167 ± 9.3***	14
6	1430	1325	120.8 ± 8.7	152 ± 5.7***	14
7	1512	1332	125.5 ± 11.1	128 ± 8.3	11
8	1666	1511	116.6 ± 7.3	112 ± 15.1	9

* Standard error of the mean.
** Differs from control at 5 percent level of significance.
*** Differs from control at 1 percent level of significance.

Adrenals analyzed on the third day of infection showed a slight but statistically significant decrease. This was followed by a pronounced increase which reached its maximum at the end of the fifth day of infection. This was followed by a decreasing trend so that the values were near control levels by the eighth day.

Total ascorbic acid determinations done on the adrenals of cockerels on the third, fifth, and eighth day of infection revealed similar changes (table II). Al-

TABLE II. *The effect of cecal coccidiosis upon the adrenal total ascorbic acid of White Rock cockerels.*

Day of infection	Body weight (gm)		Adrenal weight (mg)	Adrenal ascorbic acid (mg percent)	No. of birds
	Pre-infection	At sacrifice			
0		855	82.6 ± 3.1*	156 ± 4.0*	20
3	709	859	83.2 ± 2.7	147 ± 3.6	25
5	667	716	91.3 ± 4.4	207 ± 8.8***	21
7	621	671	87.5 ± 4.0	160 ± 5.4	19

* Standard error of the mean.
*** Differs from control at 1 percent level of significance.

though the total ascorbic acid levels for the third day were somewhat lower than those of the controls the difference is not statistically significant. The ascorbic acid concentration observed on the fifth day showed a marked and highly significant increase and those of the eighth day were near control values.

Adrenal weights in both groups seem to show a trend toward enlargement. However, the data are difficult to interpret because of the considerable variation in body weight between groups and the loss of weight that took place as a result of the infection. The differences in absolute adrenal weight that were observed are not statistically significant (tables I, II).

Experimental Hemorrhage: Repeated bleedings resulted in a marked increase in adrenal ascorbic acid (table III). This was associated with a drop in red cell

TABLE III. *The effect of experimental hemorrhage upon the adrenal total ascorbic acid of White Rock cockerels (birds sacrificed 12 hours after bleeding).*

Treatment	Body weight (gm)	Adrenal weight (mg)	Adrenal ascorbic acid (mg percent)	Hematocrit before bleeding	(Percent) at sacrifice	Number of birds
Control—no treatment	1566	119.2 ± 6.9*	168 ± 5.7	—	28.6 ± 1.1	10
Control—heart punctured without removing blood	1720	123.8 ± 10.7	193 ± 2.7**	29.6 ± 1.3	24.6 ± 1.4	5
Repeated bleedings	1593	123.7 ± 6.8	255 ± 5.9***	28.8 ± 0.7	15.2 ± 0.5	10
Single bleeding	1536	128.5 ± 9.2	221 ± 8.3***	28.3 ± 0.7	17.1 ± 0.6	10

* Standard error of the mean.
** Differs from control at 5 percent level of significance.
*** Differs from control at 1 percent level of significance.

volume of 13.6 percent. The control group which received repeated heart punctures without removal of blood also showed some increase in ascorbic acid when compared to untreated controls. This was less pronounced than that of the group which was bled as was the drop in red-cell volume (5.0 percent).

Single massive bleedings resulted in a marked increase in adrenal ascorbic acid content within 12 hours when compared to the untreated controls (table III). The change was somewhat less than that found in the group which was bled repeatedly. The drop in red-cell volume was also somewhat less (11.2 percent).

The 12-hour period between bleeding and determination of the adrenal ascorbic acid level used in the above experiments was selected because a similar length of time transpires between the onset of hemorrhage and the maximum elevation of adrenal ascorbic acid in coccidiosis infected birds. Since it seemed possible that there might be an initial depletion of ascorbic acid taking place before the observed elevation, another series of ascorbic acid determinations were done at 3 and 6 hours after a single massive hemorrhage. The adrenals of these birds showed a slightly elevated ascorbic acid level at 3 hours and an additional slight increase at 6 hours (table IV). However, the changes are not statistically significant when compared to untreated controls.

TABLE IV. *The effect of experimental hemorrhage upon the adrenal total ascorbic acid of White Rock cockerels (birds sacrificed 3 and 6 hours after a single massive bleeding).*

Treatment	Body weight (gm)	Adrenal weight (mg)	Adrenal ascorbic acid (mg percent)	Hematocrit before bleeding	(Percent) at sacrifice	Number of birds
Control—no treatment	2398	164.2 ± 14.6*	146 ± 4.8	—	33.6 ± 1.6	6
Experimental—sacrificed 3 hrs. after bleeding	2137	153.7 ± 8.8	156 ± 5.0	35.0 ± 0.8	20.0 ± 1.1	8
Sacrificed 6 hrs. after bleeding	1975	142.7 ± 10.1	161 ± 4.1	34.5 ± 1.8	20.2 ± 0.5	6

* Standard error of the mean.

DISCUSSION

Cecal coccidiosis infections have a pronounced effect upon the host's adrenals as judged by the changes which take place in adrenal ascorbic acid. The elevation in ascorbic acid takes place at a time of maximum stress as judged by the occurrence of mortality, weight loss, and the various metabolic alterations characteristic of the cecal coccidiosis syndrome. It is at this time that the liberation of the second-generation merozoites and the resultant hemorrhage takes place. During this critical phase of the infection erythrocyte counts and the red-cell volumes are frequently depressed more than 50 percent (Natt and Herrick, 1955).

Some of the metabolic changes associated with cecal coccidiosis suggest that alterations in adrenal functions are involved. Among these are the hyperglycemia and increased insulin tolerance that have been observed (Pratt, 1940; Peterjohn, 1956). However, there will be little basis for evaluation of the role of the adrenal in the host's response to the infection until a better understanding of the physiology of the avian adrenal is obtained.

Elevation of adrenal ascorbic acid during the period of maximum stress was unexpected in the light of previous observations of depletion as a characteristic response to stress in mammals, and the general lack of response which has been found in birds. Full interpretation of these changes must await elucidation of the role of ascorbic acid in adrenal function.

An attempt is in progress to correlate ascorbic acid changes with variations in adrenal cholesterol content and corticosteroid secretion. Preliminary results lead to the conclusion that cholesterol content varies inversely with adrenal ascorbic acid in birds infected with cecal coccidiosis, and is thus depleted during the critical phase of the infection.

The finding that severe experimental hemorrhage brings about adrenal ascorbic acid elevation indicates that the observed changes in infected birds may be due to blood loss alone and not necessarily to toxic effects of the parasite. Similar conclusions have been drawn concerning the hyperglycemia associated with cecal coccidiosis (Pratt, 1940).

While ascorbic acid elevation seems to be characteristic of cecal coccidiosis infections and experimental hemorrhage in chickens, it seems possible that some ascorbic acid depletion might be present as a transient stage prior to the observed elevation. Such changes might have been missed with the sampling intervals used in this study. The finding of slightly reduced ascorbic acid levels on the third day of infection favors this possibility.

These observations give support to the concept that turnover rate is important in determining the absolute level of adrenal constituents (Elton et al, 1959; Kessler and Leathem, 1952). Thus, the net gain in ascorbic acid which was observed may be due to repletion occurring simultaneously and at a greater rate than the usual stress induced depletion. Under such a condition ascorbic acid could be playing a fundamentally identical role in situations where either depletion or elevation is the net effect of stress. Studies on the ascorbic acid levels of adrenal effluent blood might clarify the problem.

A final factor that should be considered is that hemorrhage may not be an appropriate nonspecific stress. It is possible that blood loss or one of its consequences (i.e., anoxia, hemodilution, hemopoetic stimulation) has a specific effect upon adrenal ascorbic acid metabolism. This does not seem to be true with the rat in

which there is a typical adrenal ascorbic acid depletion in response to massive hemorrhage (Sayers et al, 1945).

SUMMARY

The effect of cecal coccidiosis and experimental hemorrhage upon adrenal ascorbic acid concentrations was studied. A marked increase in ascorbic acid was observed in the adrenals of chickens with acute coccidiosis. This could be duplicated by experimental hemorrhage.

The significance of the findings are discussed in relation to the results of other studies.

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RESEARCH NOTE

AN IMPROVED METHOD OF PROPAGATING *EIMERIA TENELLA* OOCYSTS.

This division sometimes receives requests to supply cultures of oocysts of poultry coccidia and information on the best method of propogating these organisms. Since most of these requests have specified *Eimeria tenella*, the cecal coccidium of chickens, instructions for its propagation were prepared in 1954. Later, refinements in this technique were developed, which reduce the time and effort involved without impairing the results. The revised procedure follows:

(1) Inoculate coccidia-free chicks at 3 to 4 weeks of age with 2,500 to 5,000 sporulated oocysts. If not otherwise available, these oocysts may be obtained from the blind end of the ceca of birds with current or recent signs of cecal coccidiosis. The oocysts should be given per os, using a 1-cc pipette. The number of chicks used will depend on the quantity of culture desired, but 15 to 20 will yield a bountiful supply of oocysts. Younger chicks may be used, but their smaller ceca do not yield as rich a harvest for the same amount of effort. Older chicks, on the other hand, have had more opportunity to pick up natural infections with *E. tenella* and other species of coccidia.

(2) Sacrifice the inoculated chicks after 7.5 to 8 days and remove their ceca intact.

(3) Place the ceca in a petri dish which contains a 2.5 percent solution of potassium dichromate, and cut them into small pieces by means of sharp scissors. Comminute in a Waring-type blendor for 3 minutes.

(4) Screen out the larger particles of host tissue and debris by means of a moderately fine-mesh sieve (an ordinary kitchen strainer will serve if nothing better is available) and transfer the suspension to shallow dishes. Add enough tap water to cover the bottom of each dish to a depth of one-eighth to one-fourth inch.

(5) Let the culture stand at room temperature for about 5 days. Add tap water when necessary to prevent drying. The culture should be aerated by stirring two or three times a day or, preferably, by bubbling air slowly through the liquid if facilities are available. The time necessary for satisfactory sporulation cannot be stated exactly. It varies with temperature, amount of aeration, and probably other factors; periodic microscopic examination is the best method of determining progress of sporulation.

(6) When a satisfactory ratio of sporulated to unsporulated oocysts has developed, pour the material into cone-shaped sedimentation glasses to concentrate the oocysts. Let stand for about 4 hours, decant, add more water, and repeat the sedimentation process. After the supernatant fluid is removed for the second time, pour the residue into glass jars that have tight-fitting lids. Brown glass is preferable, but not obligatory. Store under normal refrigeration until needed.

(7) The strength of the culture (number of sporulated oocysts per milliliter) can be determined at the convenience of the investigator.

It may be desirable to grade the ceca, as in an experiment in which the same strain of oocysts is to be used in serial inoculations. If grading is done, open the ceca and evaluate the lesions as the ceca are placed in the petri dish (see step 3).—J. L. GARDINER AND D. K. McLOUGHLIN, *Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.*

ISOSPORA SP. (PROTOZOA: EIMERIIDAE) FROM CATTLE AND ITS RELATIONSHIP TO *I. LACAZEI* OF THE ENGLISH SPARROW*

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In the course of a survey of coccidia of cattle during the summer and fall of 1959, an *Isospora* was encountered in three herds of beef cattle in central Illinois. It was thought at first to be a new species, since it did not resemble any other species of *Isospora* described from ruminants nor did it resemble the published descriptions of any species of *Isospora* found in other animals which live in contact with cattle. These included *I. bigemina, I. rivolta,* and *I. felis* of the dog and cat, *I. suis* of the pig and *I. lacazei* of passeriform birds.

Later, however, an opportunity presented itself for examining the oocysts of *I. lacazei* from the English sparrow, and it was found that they differed from the published illustrations and descriptions of this species and that they were practically indistinguishable from the form found in cattle feces. In addition, a review of the literature revealed that all the figures and descriptions of *I. lacazei* except those of Scholtyseck (1954) were apparently based on infections of hosts other than the English sparrow. Hence it was felt worthwhile to redescribe this coccidium from this host.

MATERIALS AND METHODS

Fecal samples were examined from 12 calves on one farm, 31 cattle on a second, and 11 on a third. They were collected from the first herd in August 1959. All the animals in this herd were 6-month-old one-fourth Brahma (*Bos indicus*) by three-fourths Hereford (*Bos taurus*) hybrids.

Fecal samples were collected from the second farm on two dates 6 weeks apart. The first group of samples was taken in August 1959 from 10 one-fourth Brahma by three-fourths Hereford, 6-month-old hybrid calves which had been shipped in from Texas 6 weeks previously. The second group of samples was taken in September 1959 from 14 calves in this same group, from a Hereford bull, and from six one-fourth Brahma by three-fourths Charolaise (*Bos taurus*) hybrid calves which had been shipped in from Texas 5 weeks previously, and which had been placed on the pasture vacated by the Brahma-Hereford hybrids.

Eleven fecal samples were collected from the third farm in October 1959. The animals in this herd were Polled Herefords. *Isospora* was first seen in this herd by Dr. Joseph Szanto of the College of Veterinary Medicine, University of Illinois.

The first two farms were located near Monticello, Illinois and the third farm near St. Joseph, Illinois.

Composite fecal samples were obtained from a group of caged, recently captured English sparrows (*Passer domesticus*) in December 1959 thru the courtesy of Dr. David D. Myers, College of Veterinary Medicine, University of Illinois.

Prior to examination, the fecal samples were placed in 2.5 percent potassium bichromate solution, mixed thoroughly, placed in a thin layer in a petri dish, and kept at room temperature for a week or more in order to allow the coccidian oocysts to sporulate. The samples were then stored in a refrigerator. They were examined microscopically after sugar flotation. A Leitz

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Ortholux microscope with apochromatic objectives was used for critical examinations and drawings. Some bovine samples were examined prior to sporulation in searching for positive animals.

RESULTS

Isospora sp. from Cattle

Isospora was found in a total of six animals from the three farms. It was found in one of the 12 Brahma-Hereford calves on the first farm, in two of the 10 Brahma-Hereford calves in the first collection and in two of the 6 Brahma-Charolaise calves in the second collection on the second farm, and in one 8-month-old steer from among the 11 Polled Herefords on the third farm.

Description: Oocysts (fig. 1) usually subspherical, occasionally spherical.

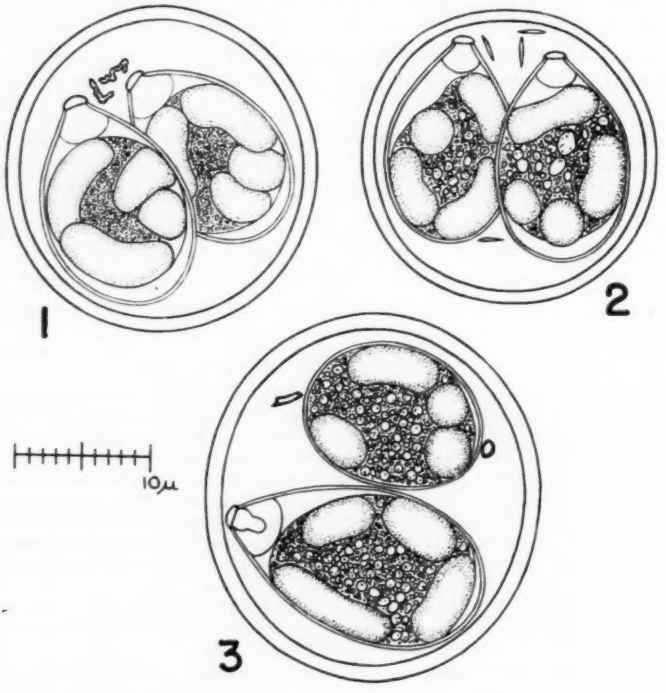

FIGURE 1. Sporulated oocyst of *Isospora* sp. from cattle, × 2000.
FIGURES 2, 3. Sporulated oocysts of *Isospora lacazei* (Labbé, 1893) from *Passer domesticus*, × 2000. One of the sporocysts in figure 3 is oriented so that the Stieda body is not visible.

Forty-nine oocysts from six animals measured 21 to 33 by 20 to 32 microns with a mean of 26.7 by 24.9 microns. Their length-width ratios ranged from 1.0 to 1.2 with a mean of 1.07. Oocyst wall smooth, colorless, pale lavender or pale yellowish, composed of a single layer about 1 micron thick. In some oocysts, the wall appeared to be lined by a thin membrane; in others, no membrane could be seen. Micropyle absent. Oocyst residuum absent. Several irregular, refractile, polar granules present. Sporocysts lemon-shaped, quite thick-walled. Nine sporocysts measured 14 to 20 by 10 to 12 microns with a mean of 16.6 by 11.0 microns. Their length-

width ratios ranged from 1.4 to 1.7 with a mean of 1.51. Sporocyst Stieda body a button-shaped cap, with a dependent globular, hyaline mass protruding into the interior of the sporocyst. Sporocyst residuum finely granular. Sporozoites appear sausage-shaped, not arranged in any particular order in sporocyst. Sporocyst residuum and sporozoites enclosed in a membrane, forming more or less of a ball within the sporocyst.

Remarks: Basanov (1952) described *Isospora aksaica* from 12- to 30-day old calves in Kazakhstan. His paper is, apparently, not available in this country, and the only description and figures we have seen are those given by Orlov (1956) in a book which is itself apparently quite rare here. According to this description, *I. aksaica* has spherical oocysts measuring 26 by 26 microns which are dark silver under low magnification and light grey with a pinkish tinge under high. The oocyst wall is smooth, 1.6 microns thick, and double-contoured. Its outer layer is light blue and its inner one greenish, dingy rose. (In the illustrations, only a single layer is shown.) The sporocysts are said to be ellipsoidal or spherical and to measure 22 by 15 microns, but in one figure what might be a Stieda body is shown. The sporozoites are spherical, bean-shaped or ellipsoidal and measure 15 by 11 microns. Nothing is said in the description about a micropyle, oocyst residuum, sporocyst residuum or polar granules. The first three are absent in the illustrations, but what might or might not be two polar granules are shown in one figure. In addition, two of the "sporozoites" (one in each sporocyst) appear to contain two "infra"-sporozoites each; this may have been due to an error in drawing.

The *Isospora* found in Illinois cattle differs from *I. aksaica* in the size and shape of its sporocysts, in having sporocyst residua, and perhaps in other ways as well.

The only other report of *Isospora* in cattle appears to be that of Cooper and Gulati (1926). They found *Isospora* oocysts in five of 181 cattle (*Bos indicus*) which they examined in Assam. Two of the animals were calves, two were cows and one was a heifer. The oocysts were rare in all the animals. Cooper and Gulati were unable to measure the oocysts or to make a detailed study of them at the time of their discovery. They preserved the fecal samples in formalin, but could find no *Isospora* when they examined them again 8 months later. However, they stated that the oocysts were quite round in outline and that the sporoblasts were formed within 24 hours. There is nothing in this meager description which differs from that given above, and it is not unlikely that Cooper and Gulati may have seen the same form.

The only other species of *Isospora* described from ruminants are *I. rangiferis* Yakimoff, Matschoulsky, and Spartansky, 1937, from reindeer (*Rangifer tarandus*) on the Kola Peninsula, USSR; *I. capreoli* Svanbaev, 1958, from the roe deer (*Capreolus capreolus*) in Kazakhstan, USSR; and *I. orlovi* Tsygankov, 1950, from the camel in Kazakhstan.

I. rangiferis was described in some detail and illustrated by Yakimoff, Sokoloff, and Matschoulsky (1936) before being named by Yakimoff, Matschoulsky, and Spartansky (1937). Our bovine *Isospora* resembles it somewhat, but differs in having rounder oocysts and sausage-shaped rather than comma-shaped sporozoites, and in the shape of its Stieda body; in addition, the figure of *I. rangiferis* showed a round body in the sporozoites, perhaps the nucleus, which was not seen in the sporozoites of our bovine *Isospora*. The latter differs from *I. capreoli* in oocyst shape and

color, in having a one- rather than a two-layered oocyst wall, in lacking a micropyle and oocyst residuum, and in having an oocyst polar granule and a sporocyst residuum. Our bovine *Isospora* differs from *I. orlovi* in having much rounder oocysts and in having oocyst polar granules and characteristic sporocyst Stieda bodies.

Isospora lacazei (Labbé, 1893)

Many oocysts of this species were found in English sparrow feces.

Description: Oocysts (figs. 2, 3) usually subspherical, sometimes spherical or ellipsoidal. One hundred oocysts measured 18 to 29 by 17 to 27 microns with a mean of 24.1 by 22.1 microns. Their length–width ratios ranged from 1.0 to 1.3 with a mean of 1.09. Oocyst wall smooth, colorless to pale yellowish, 0.8 to 1.2 microns thick, composed of a single layer which sometimes appears to be lined by a membrane. Micropyle absent. Oocyst residuum absent. One to several oocyst polar granules present in 73 percent of 100 oocysts, usually splinter-like, but sometimes rounded. Sporocysts ovoid to lemon-shaped, rather thick-walled, sometimes with smaller end drawn out. Fifty sporocysts measured 13 to 20 by 9 to 12 microns with a mean of 16.1 by 10.4 microns. Their length–width ratios ranged from 1.4 to 1.8 with a mean of 1.55. Sporocyst Stieda body a button-shaped cap, with a dependent, globular, hyaline body protruding into the interior of the sporocyst. This body sometimes contains a cylindrical core extending part way down from the Stieda body. Sporocyst residuum present, finely granular. Sporozoites sausage-shaped, not arranged in any particular order within the sporocyst. Sporocyst residuum and sporozoites enclosed in a membrane, forming more or less of a ball within the sporocyst.

Remarks: *Isospora lacazei* has been reported from a large number of passeriform and other birds (Boughton, 1938; Scholtyseck, 1954, 1956; Ryšavý, 1954, 1956), but whether all the forms reported under this name actually belong to the same species remains to be determined. Almost all of the published descriptions and illustrations leave so much to be desired that a complete reinvestigation of the isosporas of these birds is needed to clear up the situation. In the discussion which follows, all the birds belong to the order Passeriformes unless it is stated otherwise.

I. lacazei was first described by Labbé (1893) under the name *Diplospora lacazii* from the European goldfinch (*Carduelis carduelis*), European skylark (*Alauda arvensis*), and other unnamed birds in France. Later Labbé (1896) reported it from the domestic sparrow (*Passer domesticus*) and other birds. At the same time he emended the spelling of the specific name to *lacazei*. This is the correct spelling. It honors Felix Joseph Henri de Lacaze-Duthiers (1821–1901), and *lacazii* is clearly a lapsus calami.

In his original description Labbé (1893) stated that the oocysts of this species were spherical, 23 to 25 microns in diameter, and contained two piriform or fusiform sporocysts each containing four sporozoites. There was no oocyst residuum.

In the same paper Labbé (1893) described *Diplospora rivoltae* as a new species from the chaffinch (*Fringilla coelebs*), red-backed shrike (*Lanius collurio*), continental blue titmouse (*Parus caeruleus*), and other unnamed birds in France. He said that the only difference between these species was that the oocysts of *I. rivoltae* were 16 to 18 microns in diameter. Both species could cause either chronic or acute infections. Experimentally infected chaffinches and goldfinches died in two or three

days, and their intestines were packed with coccidia in all stages of development.

Later Labbé (1896) synonymized *I. rivoltae* with *I. lacazei,* stating that he had found oocysts of intermediate size in other hosts. He gave no figures in either paper or in his later review (Labbé, 1899).

Sjöbring (1897) reported *Isospora* in 11 species of birds in Sweden. Nine were members of the order Passeriformes, but no species of *Passer* was among them. He figured oocysts from the red-backed shrike (*Lanius collurio*), and his description accords with them, but he stated that the coccidia from the other birds looked much the same. These oocysts were spherical or sometimes oval, with a double-contoured wall. They contained some greenish polar granules and two flask-shaped sporocysts, each with a stopper-like Stieda body. Each sporocyst contained four to six sickle-shaped sporozoites and some granular residual material. His illustrations showed five sporozoites. There was no micropyle or oocyst residuum. Sjöbring used two names for this form. At the beginning of his paper he called it *"Isospora Passerum* n.n.," and later on, *"Isospora communis passerum."* The former name takes precedence. He remarked, however, that except for the number of sporozoites, there was no great difference between his coccidia and Labbé's *Diplospora lacazei* and *D. rivoltae.* Sjöbring thought that there might be more than one species of *Isospora* in these birds, but that cross-transmission studies would be needed to find this out. Later writers have ignored or discounted his statement about the number of sporozoites and have synonymized his species with *I. lacazei.*

Hosoda (1928) was apparently the first to describe *Isospora* from a member of the genus *Passer,* in this case *P. montanus saturatus.* His figures were reproduced by Becker (1934), and have been the standard for *I. lacazei* ever since. His oocysts were ellipsoidal, ovoid or subspherical, with a three-layered wall composed of a thin exomembrane, a thin endomembrane, and a heavier intermediary layer. Hosoda did not give any measurements. There was no micropyle, oocyst residuum or oocyst polar granule. The sporocysts were broadly rounded at one end and nipple-like at the other, with a prominent Stieda body which was not merely a thickening of the sporocyst wall. Each sporocyst contained four sporozoites and a sporocyst residuum.

Boughton (1930, 1933, 1937) found that *Isospora* was extremely common in the English sparrow in Wisconsin, and did a great deal of experimental work on it. He considered it to be *I. lacazei.* He did not describe or draw the oocysts, but he did measure a large number of them and found that early in the course of an infection they ranged from 23 to 34 microns by 22 to 34 microns with a mean of 26.9 by 25.8 microns, while later on forms as small as 17 microns were present.

Henry (1932) reported finding *I. lacazei* in 50 percent of 140 passeriform birds, most of which she did not name, and also in a few birds of other orders in California. She described the oocysts as often subspherical, but frequently oval or ovoid, with a wall composed of three layers of which the middle one was comparatively thick. The oocysts ranged from 16 to 32 by 13 to 29 microns; those in one English sparrow measured 22 to 32 by 19 to 26 microns and were mostly ovoid. The micropyle was usually inconspicuous or could not be seen at all. There were one or a few polar granules but no oocyst residuum. The sporocysts usually contained a rather large residual body, and frequently a knob at one end of the sporocyst was prominent.

Henry gave photomicrographs of sporulated oocysts from the robin(*Turdus migratorius*) and flicker (*Colaptes cafer*) (order Piciformes). That of the former was ovoid and that of the latter spherical. The sporocyst Stieda body of the robin form resembled that which we have described above from the sparrow, but that of the flicker form was not in good enough focus to be sure of this. Henry did not illustrate oocysts from the sparrow or describe them specifically.

Scholtyseck (1954) found two sizes of oocysts in the house sparrow (*Passer domesticus*) in Germany and assigned separate names to them. He did not describe them in detail, but he did illustrate them, and the following descriptions are taken in part from his figures. The larger and less common species, with spherical or subspherical oocysts 30 to 39 microns in diameter with a mean of 33.6 microns, he called *I. lacazei*. Its oocysts had no micropyle or oocyst residuum but contained two polar granules. The sporocysts were about 30 microns long, broadly ovoid, with a trace of a Stieda body (a slightly thickened sporocyst wall) at the small end, and contained four sporozoites, each with a clear "vacuole" at one end, and a small amount of residual material. The smaller and more common species, with spherical or subspherical oocysts 18 to 30 microns in diameter with a mean of 24 microns, he called *I. passerum* (but he labelled it *I. lacazei* in his illustration). Its oocysts had no micropyle or oocyst residuum but contained two polar granules. The sporocysts were about 15 microns long, irregularly elongate ovoid, with a trace of a Stieda body (a slightly thickened sporocyst wall) at the small end, and contained four sporozoites, each with a clear "vacuole" at one end, and a small amount of residual material. Later Scholtyseck (1956) reported *I. lacazei* from 40 species of birds of several orders, and *I. passerum* from *Passer domesticus* and from two crows, *Corvus frugilegus* and *C. corone*.

Ryšavý (1954) described *I. lacazei* from various birds, including the English sparrow, in Czechoslovakia. Its oocysts were spherical or sometimes subspherical, thin-walled and had no micropyle or oocyst residuum. Its sporocysts were spherical and contained a large residuum. He stated that the morphology of the oocysts changed with different hosts, but did not give separate descriptions from each host. Oocysts from the English sparrow measured about 18 to 29 microns in diameter with a mode of about 22 microns.

The *Isospora* from *Passer domesticus* in Illinois described in the present paper differs in one way or another from all the above forms except Labbé's (1893) description of *I. lacazei*, but the latter was so sketchy that it is of relatively little value for comparative purposes. The Illinois *Isospora* resembles that described by Sjöbring (1897) from *Lanius collurio* in having oocyst polar granules and in the shape of its sporocysts and Stieda body, but not in the number of sporozoites per sporocyst. It differs from the form described by Hosoda (1928) from *Passer montanus* in having oocyst polar granules. It differs from the form described by Henry (1932) from various birds in always lacking a micropyle. It differs from the form described by Ryšavý (1954) from various birds in the shape of its sporocysts, which are not spherical. It differs from both the forms described by Scholtyseck (1954) from *Passer domesticus* in the appearance of the Stieda body and in lacking "vacuoles" in the sporozoites; in addition, it is smaller than his *I. lacazei*.

Two notable features of our *Isospora* from *P. domesticus* are the shape of the Stieda body and the fact that the sporocyst residuum and sporozoites are enclosed in a membrane, forming more or less of a ball within the sporocyst. The second

character was not mentioned in any earlier description or shown in any earlier drawings. Indeed, all the latter showed that there was no such membrane. As to the Stieda body, the drawings of Sjöbring (1897) of the form from *Lanius collurio,* of Hosoda (1928) of the form from *Passer montanus* and the photomicrographs of Henry (1932) of the forms from *Turdus migratorius* and possibly *Colaptes cafer* resemble the Illinois sparrow form well enough, but Ryšavý (1954) described and illustrated the sporocysts as spherical, i.e., without a Stieda body at all, and Scholtyseck's (1954) drawings showed only a slight thickening of the sporocyst wall.

All the above descriptions of *"Isospora lacazei"* are not alike, and some of them are directly contradictory. The fact that most of them were based on oocysts from different hosts may account for some of the discrepancies; if these differences turn out to be valid and consistent, it would mean that several species of *Isospora* are involved. On the other hand, many of the descriptions and illustrations are so sketchy and unsatisfactory that one cannot be sure exactly what the authors saw. Carefr', detailed, complete descriptions and accurate drawings of all these coccidia from different hosts are badly needed before valid comparisons can be made, and careful cross-transmission studies must also be carried out.

In the meantime, the situation regarding the names and number of species of *Isospora* in these birds can be clarified somewhat.

Ryšavý (1956) maintained that the only differences between *I. lacazei* and seven other species of *Isospora* which had been described from birds were in the size of their oocysts and their occurrence in different hosts, and lumped them all together under *I. lacazei.* He said that the only reason they had been assigned to different species was that (in translation) "the authors were obviously completely under the influence of phylogenetic determinism and described the new species, not on the basis of morphological characteristics or an analysis of ecological factors, but only on the basis of finding oocysts in a host not previously mentioned in the literature." Without bothering to refute this statement in detail, it is enough to mention that one of the species he synonymized with *I. lacazei* was *I. buteonis* Henry, 1932 from various hawks and owls (order Falconiformes). This is a *bigemina* type of *Isospora,* morphologically entirely different from *I. lacazei.*

Scholtyseck's (1954) two forms from the English sparrow might turn out to be separate species, but the names he assigned to them are not above criticism. He assigned the name *I. lacazei* to the larger form with oocysts 30 to 39 microns in diameter, and the name *I. passerum* to the smaller form with oocysts 18 to 30 microns in diameter. However, the oocysts of Labbé's *I. lacazei* were 23 to 25 microns in diameter (within the size range of Scholtyseck's smaller form), and no one else has reported oocysts from the English sparrow more than 34 microns long. Hence, if either of Scholtyseck's forms is to be assigned a new name, it should be the larger one.

The name *Isospora passerum* was first used by Sjöbring (1897) for the coccidia he described from 11 species of birds, none of which belonged to the genus *Passer.* It has passed into synonymy with *I. lacazei,* and to resurrect it for a form which differs morphologically from the original one and which is from a different host is not permissible.

The best way to minimize confusion would be to retain for the present only the name *I. lacazei* for all those forms of *Isospora* from passeriform birds customarily referred to this species and for morphologically similar forms from other birds,

recognizing that it may very well be a complex of several species. If future investigations justify it, one or more species might be split off from this complex, and when this is done they should be given new and uncompromised names.

DISCUSSION

There is always a question in the case of coccidia which are found in feces alone whether they are a true parasite of the animal in which they were found or whether they were merely passing thru its digestive tract. Perhaps the most famous case of mistaken identity is that of *Eimeria wenyoni, E. oxyspora* and *E. snijdersi,* all of which Dobell (1919, 1921) described as new species from man but which turned out to be coccidia of herrings, sprats, mackerel, and sardines (Wenyon, 1926).

In the present case, the fact that the oocysts described from bovine feces were found on three widely separated farms and at two different times on one of them, coupled with the fact that they differed from the published descriptions of all other coccidia which might have been contaminants, suggested that they were a true bovine species. Lack of information regarding the endogenous stages was not a serious matter, since these are known for only four of the 10 commonly accepted species of *Eimeria* of cattle.

However, the marked morphological similarity between the bovine and sparrow Isosporas raises the question whether they are the same. The only notable morphological difference between them is that the cylindrical core in the dependent part of the Stieda body illustrated in figure 3 was never observed in the bovine form. However, it was seen in only a small minority of the sparrow coccidia, so this difference is not decisive. The similarity of all other structures, and especially the presence in both forms of the same two notable features (the structure of the Stieda body and the membrane enclosing the sporozoites and sporocyst residuum), suggests that the two coccidia may be the same. Final proof one way or the other, however, will depend upon the results of experimental studies in cattle.

The feed of cattle which are kept in a barn or are fed concentrates in a trough could be easily contaminated with sparrow droppings. In a steer which produces about 20 pounds of feces per day, the presence of one oocyst per gram of feces would represent contamination of the feed with about 9000 oocysts (assuming that the oocysts were mixed uniformly with the ingesta and passed thru the animal unchanged). Assuming again that a flotation was carried out with about 2 g of feces and that about 10 percent of the oocysts present were recovered, one can calculate that every oocyst found would represent an initial contamination of a day's feed with about 45,500 oocysts. Since Boughton (1933) quite frequently obtained counts of 200,000 to 2,000,000 oocysts per gram of dried sparrow feces, it is quite likely that sparrow coccidia could be detected in a calf's feces if it ate only a single fecal deposit from a single sparrow in the course of a day. The largest number of coccidia we found in any one flotation preparation from a calf was 14 which, on the basis of the above assumptions, would represent contamination of a day's feed with about 640,000 oocysts.

SUMMARY

A species of *Isospora* with subspherical to spherical oocysts 27 by 25 microns in diameter was observed in the feces of six beef cattle on three farms in central Illinois. It was thought at first to be a new species, but a comparison with the

oocysts of *I. lacazei*, the common coccidium of the English sparrow (*Passer domesticus*), revealed that the two forms were practically indistinguishable, so that the oocysts found in bovine feces may well be those of *I. lacazei*.

The literature on the taxonomy and morphology of *I. lacazei* from passeriform and other birds is reviewed and its oocysts are redescribed from the English sparrow. They measure 18 to 29 microns by 17 to 27 microns with a mean of 24 by 22 microns. Future work may show that several species are actually now being lumped under this name.

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RESEARCH NOTE

ASSOCIATION OF *GIARDIA LAMBLIA* WITH DISEASE.

Recent studies of enteric disease and its control in several communities near Phoenix, Arizona, included observations of human intestinal parasites. This note provides an estimate of the frequency with which persons who have infections of *G. lamblia* report diarrhea as compared to individuals not infected with *Giardia*. Pathogenicity is sufficiently regarded with reference to *G. lamblia* insofar as children are concerned that treatment of all such infections is recommended (Jung, 1958, J. Pediatrics **53:** 89–98).

The data presented here were compiled from three separate studies carried out in Maricopa County, Arizona, during 1958 and 1959. The first study involved the family contacts of children who were admitted to a local hospital with diarrheas due to enteropathogenic *Escherichia coli* or salmonellae. Histories of illness during the preceding month were obtained from all family contacts. Rectal swab and passed stool specimens for bacteriologic examinations were collected from all family contacts when possible and a portion of the passed stool specimen was examined in the parasitology laboratory.

The second and third studies were random sample surveys of two communities in the Phoenix area. As in the first study, histories of illness during the previous month were obtained for all the members of each family visited. Passed stool specimens were requested from each individual and these too were examined both bacteriologically and parasitologically.

All fecal samples were examined in the laboratory by three methods: (1) direct smear, (2) formalin-ether concentration, and (3) staining with either iron-hematoxylin or Wheatley's trichrome.

A total of 400 persons was examined parasitologically in the three studies. Of these, 38 (9.5 percent) were found to be infected with *G. lamblia*, as shown in table I. The attack rate

TABLE I. *Attack rates for diarrhea among individuals infected and uninfected with* G. lamblia.

	Total	Ill	Attack rate (percent)
Infected with *G. lamblia*	38	8	21.1
Uninfected with *G. lamblia*	362	27	7.5
TOTAL	400	35	8.8

for diarrhea among infected individuals was 21.1 percent, while the rate for those who were not infected was 7.5 percent. The probability that the differences observed here are due to chance alone is less than 1 percent. The age and sex of the cases and the time elapsed between onset of illness and collection of specimens are presented in table II. The preponderance of males is of

TABLE II. *Age, sex, and time elapsed between onset of illness and stool examination for the eight giardial diarrheas.*

Case No.	Age	Sex	Time elapsed (days)
N-1-3	12	M	6
N-4-1	30	M	4
W-4-6	6	F	30
W-9-4	3	M	1
22-227-6	3	M	5
F-5-2	3	M	10
F-15-3	1	M	7
F-33-2	27	F	3

interest since the sex distribution among the individuals examined and those infected with *Giardia* was approximately equal. The median age of the cases was 4.5 years with a range of from 1 to 30 years.

The present investigation confirms a strong association between infections of *G. lamblia* and diarrhea since more than one-fifth of the individuals infected with *Giardia* recently had been ill. A relationship of cause and effect between giardiasis and diarrhea is not necessarily demonstrated by these observations. Nevertheless, diarrhea was reported much more frequently among persons with *G. lamblia* than among those not so infected. Other studies on communities in the same area disclosed that approximately 18 percent of individuals observed with illness were infected with *Giardia* (Petersen et al, to be published). By comparison, in the same studies the total incidence of the enteropathogenic bacteria, *Shigella, Salmonella,* and pathogenic serotypes of *Esch. coli,* was practically identical with that of *G. lamblia.* Investigations will be continued to gain further information on the significance of *G. lamblia* in the region.—F. J. PAYNE, F. O. ATCHLEY, M. A. WASLEY AND M. E. WENNING, *Technology Branch, Communicable Disease Center, Bureau of State Services, Public Health Service, U. S. Department of Health, Education, and Welfare, Phoenix, Arizona.*

PREVALENCE OF *TRICHOMONAS VAGINALIS* AMONG FEMALE PRISON INMATES AND INDIGENT PRENATAL PATIENTS IN THE DETROIT AREA

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Numerous published reports (Cf. Burch et al, 1959; Feo, 1956a; Feo, 1956b; Trussell, 1947) attest that the prevalence of *Trichomonas vaginalis* is likely to be high among women of realtively low socioeconomic status. The present study, which confirms a high prevalence in two such groups of women in the Detroit area, provided an unusual opportunity to observe in one of the groups the relationships of certain factors to parasite prevalence. This was a group of prisoners composed of substantial numbers of white and colored inmates who were housed without racial segregation and were comparable as to age and period of detention. These circumstances suggested that the separate influences of race, age, and duration of imprisonment on parasite prevalence might be studied. Several authors (Cf. Burch et al, 1959; Trussell, 1947) have reported higher prevalence in colored than white women and a relationship between age and prevalence, but usually the subjects were more heterogeneous than in the present situation with respect to many factors that might be influential other than simply race or age.

The other group was composed of a smaller number of indigent prenatal outpatients.

MATERIALS AND METHODS

The subjects were 405 female inmates at the Detroit House of Correction, Plymouth, Michigan, and 183 prenatal out-patients at Herman Kiefer Hospital, Detroit. The former were unselected except for the factor of being institutionalized. The latter were mainly indigent colored patients who sought help because of vaginal symptoms.

A single specimen was examined from each patient in the following manner. Vaginal material, collected on a sterile cotton swab, was inoculated into a tube containing 10 ml of fresh CPLM medium (Johnson and Trussell, 1943) and penicillin G (approximately 500 micrograms per ml) was added to suppress bacterial growth. The inoculated tubes were protected from extreme cold and delivered to the laboratory within a few hours. Upon receipt in the laboratory, they frequently were examined immediately for motile trichomonads. If circumstances did not permit immediate examination or if trichomonads were not demonstrable, the tubes were incubated at 37 C and examined at least three times during the next 5 days. The examinations were made by searching an entire coverslip (22 by 22 mm) preparation under 100 × magnification and studying suspicious bodies under 430 × magnification. The samples were identified only by number during the course of the laboratory examinations.

RESULTS AND DISCUSSION

Prison Inmates. Vaginal trichomoniasis was found in 62 percent of the 405 prison inmates. After all specimens had been examined, the inmates were tabulated in terms of parasitization, race, age, and period of detention (table I). By inspection of the data it will be noted that except for the 36- to 40-year age group the ratio of parasitization was slightly higher in each age category (confinement periods pooled) and in each confinement category (ages pooled) among colored than among white inmates.

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TABLE I. *Distribution of prison sample by race, age, and period of confinement; number found parasitized/total number examined.*

Age (yr.)	Confinement Period (yr.)								Sum		Proportion		C/W
	< 1 to 1		> 1 to 2		> 2 to 5		> 5						
	W*	C*	W	C	W	C	W	C	W	C	W	C	Ratio
17–20	6/9	12/14	2/6	0/1	1/2	0/1	—	—	9/17	12/16	0.53	0.75	1.42
21–25	8/10	15/21	6/11	10/16	1/3	11/12	—	0/1	15/24	36/50	0.62	0.72	1.15
26–30	6/8	26/46	7/11	9/13	0/3	11/16	1/1	1/1	14/23	47/76	0.61	0.62	1.01
31–35	6/9	15/18	6/12	10/12	4/6	13/16	0/1	1/1	16/28	39/47	0.57	0.83	1.45
36–40	7/12	7/10	3/5	2/5	3/4	4/10	0/3	1/2	13/24	14/47	0.54	0.52	0.96
41–45	2/6	0/1	0/1	4/4	4/5	—	1/3	1/3	7/15	5/8	0.47	0.62	1.34
46–50	5/7	2/2	1/2	1/1	1/2	5/6	1/2	0/2	8/13	8/11	0.62	0.73	1.18
51–79	1/6	1/1	1/1	0/1	1/3	0/3	0/5	3/6	3/15	4/11	0.20	0.36	1.82
Sum	41/67	78/113	26/49	36/53	15/58	44/64	3/15	7/16	85/159	165/246			
Proportion	0.61	0.69	0.53	0.68	0.54	0.69	0.20	0.44			0.53	0.67	
W/C	1.13		1.28		1.28		2.19						1.26 (P = 0.01)
Grand Sum									250/405		0.62		

* W = white, C = colored.

Chi-square analyses of the data with particular reference to race contrast yielded the following results. (1) Discrepancy in race contrast among age categories was insignificant (second order interaction $\chi^2_{(7)} = 2.59$; $P = 0.9$). (2) Discrepancy in race contrast among confinement-period categories was surprisingly small (second order interaction $\chi^2_{(3)}$ was within arithmetic rounding off error; $P = 0.99$). (3) Overall prevalence was significantly higher among colored (67 percent) than among white (53 percent) inmates ($P = 0.01$).

The raw data also suggest that the prevalence of infection was unusually low among the oldest inmates and among inmates imprisoned for the longest periods of time. The numbers of the oldest inmates were not equal across confinement periods, and vice versa. Hence, for a conservative appraisal of the influence of either factor, the extreme group of the other factor was excluded.

Analysis then showed the lower prevalence in the oldest age group (51 to 79 years) to be significant ($P = 0.02$), without evidence of race difference in this respect ($P = 0.6$). No infections were detected among 11 women that were over 60 years of age.

The lower prevalence of infection among inmates confined for 5 years or more was also significant ($P = 0.04$), without evidence of race difference in this respect ($P = 0.8$). Stability of infection prevalence up to 5 years of confinement, with falling off thereafter, suggests that the prison conditions per se were not a prime factor in the overall high prevalence of trichomoniasis. This is in accord with relatively good hygienic conditions in the institution; shower stalls rather than tubs were used for bathing, and douches were not permitted.

Exclusion of cases confined beyond 5 years and those over 50 years of age thus leaves a population with a higher (66 percent) and more stable prevalence of infection. In this trimmed population, the prevalence was still significantly ($P = 0.03$) higher in colored (70 percent) than in white (59 percent) inmates. Inasmuch as the two races were integrated in housing and exposed to a common general environment, nutrition, and sexual limitations, perhaps no more closely controlled study of the race factor has been reported. It is, therefore, interesting that the race difference in prevalence is much less than previously reported (Burch et al, 1959; Feo, 1956a; Feo, 1956b). Indeed, even though the general socioeconomic level of both races was low, it would seem most reasonable to ascribe the smaller race difference seen in this study to socioeconomic differences existing before confinement. The very stability of prevalence of infection from brief confinement to periods up to 5 years suggests preconfinement prevalence of infection to be an important factor. Burch et al (1959) suggested that a similar prevalence among certain groups of white and colored women in their study might be related to the similar socioeconomic status of the two groups.

Many of the prisoners had vaginitis, but it is beyond the scope of this paper to attempt an analysis of the relationship between their symptoms and the presence of trichomonads.

Indigent outpatients. In the 183 prenatal outpatients *T. vaginalis* was found in 61 percent. No attempt was made to analyze the relationships of age and race to parasite prevalence in these patients.

Critique of methods. Among the 199 preparations examined prior to incubation 61 percent were positive for trichomonads. Specimens found positive by this method undoubtedly would have had demonstrable organisms in conventional direct exam-

inations. Only 6.5 percent of the samples that were negative prior to incubation became positive when incubated at 37 C. It should be pointed out, however, that this is scarcely a true measure of the value of cultural methods because the specimens were not kept at optimal temperatures for *T. vaginalis* (Johnson and Trussell, 1944) until incubated.

Finally, it should be emphasized that the foregoing results based on a single specimen examined under less than optimal conditions probably underestimate by a considerable margin the true prevalence of the organism in the two population groups. Although the procedures used in this work were less than ideal, the present results indicate that they would be useful in other situations when a compromise in diagnostic procedures must be made.

SUMMARY

Single specimen examinations on 405 women prisoners and 183 prenatal patients (largely colored) showed respective *Trichomonas vaginalis* prevalencies of 62 and 61 percent.

The data on prison inmates were analyzed as to the influence on prevalence of a particular variable relatively uncomplicated by other possibly influential variables. The analyses indicated that the prevalence of infection among prisoners varied in the following respects: lower in 51- to 79-year age groups than in 17- to 50-year age groups; lower in inmates confined for more than 5 years than in those confined for shorter periods; and lower in white than colored. However, it appears from this study and other evidence in the cited literature that the more nearly equivalent the socioeconomic conditions the smaller the difference in prevalence of infection between the two races.

ACKNOWLEDGEMENTS

The authors are indebted to Mr. Albert Shapiro, Dr. Joseph A. Cortez, Mrs. Bernice Fountain (Detroit House of Correction), and Dr. John Washington (Herman Kiefer Hospital) for cooperation in this work; to Miss Priscilla McClay (Parke, Davis and Co.) for assistance in compiling the data; and to Dr. C. V. Winder (Parke, Davis and Co.) for statistical analysis of the data.

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THE BIOLOGY OF A SPECIES OF *NOSEMA* (SPOROZOA: MICROSPORIDIA) PARASITIC IN THE FLOUR BEETLE *TRIBOLIUM CONFUSUM*

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The flour beetle, *Tribolium confusum,* has been used for several years for population genetics research at Purdue University. In the spring of 1959, it came to the writer's attention that a disease had affected the beetles to the extent that entire colonies were so reduced as to become invalid for statistical consideration.

The beetles died mostly during the larval and pupal stages and then largely toward the beginning and end of those stages. Death of young larvae followed the development of large darkened areas in their fat bodies which could be detected through the body wall. Older infected larvae became distended with spores and acquired a characteristic milky appearance which was particularly noticeable because the normal folds of the exoskeleton disappear with the swelling of the body. Microscopic examination revealed massive infection with a microsporidian which was identified as a species of *Nosema,* the biology of which was investigated further.

METHODS

Flour beetles were maintained in bottles with whole wheat flour and 5 percent brewers yeast, kept in an incubator at 33 C. with elevated humidity.

Gross pathological changes in the host could be observed with the dissecting microscope. Stages of the parasite were studied under the oil immersion lens of the standard light microscope since that instrument was found to give better resolution than either the phase contrast or the interference microscope with double focus optics. To observe and measure living spores, they were suspended in thin films of 0.85 percent NaCl as hanging drop preparations sealed with petrolatum. That technique, similar to the one used by Gibbs (1953), was particularly favorable for study of the extrusion process because the filament frequently penetrated the edge of the suspension to move slowly across the glass which was dry except for droplets of condensed moisture. Observations of living spores were supplemented by the study of smears of infected larval tissue stained with gentian violet, picrocarmine, Giemsa's stain, silver nitrate or Heidenhain's hematoxylin.

Intracellular stages of the parasite were observed in serial sections of larvae fixed in Bouin's, Gilson's, Kahle's, or Schaudinn's fluid after the head and posterior end were removed to permit rapid entry of fluids. After dehydration with dioxan, n-butyl, or tertiary butyl alcohol, the larvae were embedded, sectioned at 5 microns, stained with Heidenhain's hematoxylin, and differentiated in picric acid. All figures, except figure 22, were drawn to the same scale and all measurements are in microns.

The writer is indebted to Professor R. M. Cable, who directed this study, and to Professor A. E. Bell for materials and equipment used to maintain populations of *T. confusum.*

OBSERVATIONS AND DISCUSSION

The principal site of infection was found to be the fat bodies, although nervous tissue occasionally contained spores. Infected larvae which managed to pupate usually developed no further, but instead, died after darkening to a color not to be confused with that appearing in the healthy pupa before the adult emerges. Larvae became infected by ingesting spores, either in dead larvae or in feed where they had

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disintegrated and released spores. Under crowded conditions, entire colonies quickly became infected and many dead animals were seen on the surface of the feed.

The specific identity of the parasite is uncertain. In the Nosematidae, the number of spores produced by each pansporoblast is used to distinguish genera, and on that basis the parasite in question belongs to the genus *Nosema*. Species have been based on characteristics of the spore and host-parasite relationships but the validity of these criteria may be questioned because of the extent to which they may vary under different conditions of observation. Walters (1958) reported that spore size may differ with the age of the host and the tissue infected, as well as the medium in which they are measured. The size and shape of spores may be affected by fixation, staining, and the liquid in which living ones are suspended, as Kudo (1924) recognized. Furthermore, there is considerable chance of error in measuring such small, highly refractile objects.

There has been a tendency to regard the microsporidians as strictly host specific with a distinct species for each host species found to harbor these parasites. However, Fantham and Porter (1913) used *Nosema apis* from hive bees to infect 13 other insects. Among them was *Bombus* sp., supposedly having its own species, *N. bombi,* with which, however, *Apis* sp. was readily infected (Steinhaus 1946). Furthermore, Dissanaike (1958) infected an oribatid mite and a tapeworm with the same species of *Nosema*. Because of uncertainty as to valid criteria for differentiating species of microsporidia, the parasite under discussion is designated as *Nosema* sp. Living spores suspended in 0.85 percent NaCl after being dried and measured before filament extrusion were 3.75 to 5.0 (average 4.44) long and 2.8 to 3.2 (average 3.0) wide; fixed and stained spores averaged 0.7 and 1.0 less in length and width respectively. After extrusion, the filament was 100 to 150 (average 125) long and the ejected sporoplasm 1.5 to 2.25 in diameter.

In stained films, the sporoplasm as seen at the end of the extruded filament has granular cytoplasm and two small oval nuclei about 0.3 in diameter, either close together or well separated. Although empty spores were found in the gut and hemocoele of sectioned larvae, no filaments were attached and no sporoplasms (planonts) were observed. Internal autoinfection was suggested by finding in the blood empty spore cases, presumably from the rupture of infected fat cells. A number of spores extruded the filament when taken from freshly killed larvae and mixed with its blood; the sporoplasm, however, was not always ejected.

The smallest meronts found in hematoxylin stained sections of the fat body were about 1.8 in diameter; the cytoplasm was granular but a distinct nucleus was not evident (pl. I, fig. 1). Multiplication of the parasite by binary fission, (pl. I, fig. 2), filled the fat cells with meronts 2.5 in diameter and with condensed central granulation (pl. I, fig. 3). The meronts develop into sporonts 3.5 in diameter (pl. I, fig. 4) and are more sparsely granular than the meront stage. Sporogony (pl. I, figs. 5 to 10) entails elongation of the sporont to become the sporoblast measuring 4.7×2.1 and characterized by the development of a darkly staining mass at each end, the two connected by strands along one side (pl. I, fig. 6). At first, there are about four such strands, but later they condense into a single central one (pl. I, fig. 7). This strand evidently splits in two while maintaining connections with the terminal masses and finally the darkly staining material forms a club-like structure (pl. I, fig. 8). With gentian violet, the large central portion of this structure stains red and is

distinct from the outer material. Further development of the spore content is obscured by stain on the inner surface of the spore case after it begins to form (pl. I, fig. 9). At this stage, the spore measures 3.5×2.1 and its final dimensions are 3.5×1.8 (pl. I, fig. 10) in sectioned larvae stained with hematoxylin. The spore case usually is smooth, but may have peculiar surface sculpturing (pl. I, fig. 21).

The process of filament extrusion has long been debated. Stempell (1909), Fantham and Porter (1914), Kudo (1916, 1925), Trappmann (1923), and Zwolfer (1926) postulated that the sporoplasm emerged from a pore in the spore case left by the detached filament. The filament was assumed to anchor the spore to the host tissue to facilitate passage of the sporoplasm into it from the spore case. It is not clear how the filament could have that function and also become detached to permit exit of the sporoplasm.

The sporoplasm was seen at the tip of the extruded filament as early as 1916 by Korke and later by Ohshima (1927, 1937) and Gibbs (1953, 1956). From their observations, it was postulated that the filament is hollow and that the sporoplasm is ejected through it into the host cell. Gibbs observed penetration of the host tissue and believed that the filament everted during extrusion; Ohshima thought that it was hollow but did not evert.

Dissanaike (1955) argued that the filament was not hollow because the sporoplasm seemed to be a continuation of it. He imagined the filament springing from the spore in a "jack-in-the-box" manner and "whipping out" the sporoplasm in the process. Apparently he did not observe spontaneous extrusion, but instead used pressure to force out the contents of the spore as did Laird (1959). Krieg (1955) diagrammed a hypothetical extrusion process in which pressure within the spore caused the filament to be spat out with the contents of a polar capsule. His drawings show no sporoplasm. Dissanaike and Canning (1957) apparently adapted Krieg's interpretation to their observations by deleting the polar capsule for which most authors find no evidence and by adding the sporoplasm to the tip of the filament. Krieg also presented an electron micrograph of entire spores with extruded filaments at 27,000× magnification as evidence of a solid, homogeneous filament 0.1 micron thick, but the photograph is at too low a magnification and too blurred to be convincing. The matter might be resolved by comparing sections of extruded and non-extruded filaments under the electron microscope at higher magnifications.

With the suspended film technic described above, the author repeatedly observed filament extrusion and sporoplasm emergence in saline solutions and water. The events in that process as observed with spores suspended in saline may be described as follows (the terms apex and base refer, respectively, to the end at which the filament emerges and the opposite pole of the spore): (1) A vacuole appears within the base of the spore, presumably with imbibition of fluid, and evidently increases pressure within the spore which becomes more rounded (pl. I, fig. 12). Filament extrusion is presaged by the appearance of a small papilla at the apex of the spore (pl. I, fig. 13), just as Thomson (1959) observed. (2) After a few seconds, extrusion begins, at first slowly as an elongation of the papilla to form an undulant thread which then quickly elongates in a corkscrew path counter clockwise from the spore case, causing it to shake vigorously. As the filament progresses, the denser peripheral substance within the spore disappears from near its base toward the apex (pl. I, fig. 14). If, during that process, movement of the spore is restricted by

a sufficiently thin film of saline to permit observation, a rippling movement at the apical surface of the vacuole can be seen. (3) Immediately after the filament is fully extruded, a small amount of substance remains within the apex of the spore (pl. I, fig. 15). Then, as the filament sharply contracts, that substance disappears (pl. I, fig. 16), evidently by flowing into the filament to appear at its tip as the sporoplasm. (4) When the sporoplasm emerges (pl. I, fig. 17), a blister forms at right angles to the tip of the filament and rapidly increases in diameter to an average of 4.6, which is greater than that of the spore (pl. I, fig. 18). The blister is fluid-filled and much smaller in salines than in water, apparently because of differences in osmotic pressure. (5) At or just before the blister attains full size, the filament again contracts sharply with a twisting motion that spins the blister and sporoplasm and draws them much closer to the spore. (6) The blister may burst (especially in water) with the sporoplasm becoming more rounded (pl. I, fig. 19). (7) Within the host, the filament may eventually detach from the spore case but was never seen to do so in vitro and is firmly attached. The penetrating ability of the filament and the firmness of its attachment was demonstrated by feeding spores to a ciliate. From a food vacuole, the filament of one spore extruded through 15 to 20 microns of the ciliate's cytoplasm to the outside and became entangled in some debris adhering to the slide. The ciliate, thus anchored, spun around and around in an effort to escape. In so doing, the spore case was drawn through the cytoplasm against the pellicle which was pulled out papilla-like until the spore case broke through and released the ciliate.

Although some aspects of filament extrusion in hanging drop preparations differ from that process when confined entirely within a fluid medium, the optical properties and slow movement of the filament across the glass from the edge of the liquid facilitate interpretation of the extrusion process (fig. 1). The filament extrudes quickly to the edge of the suspended drop, penetrates its surface film, and moves much more slowly across the glass. During that process, there is no rebound of the spore case as would be expected if extrusion involved the projection of the filament in the manner that Dissanaike (1955) conceived. As the filament moves in a sinuous path across the glass, it is clearly seen to lengthen only by advancement at the tip; the ejected sporoplasm is accompanied by a thin fluid and the blister does not form. To judge from its refractivity, the filament is turgid and does not adhere as closely to the glass before the sporoplasm is ejected as afterward. Thus pressure due to the imbibition of water is believed to be responsible for extrusion of the filament and ejection of the sporoplasm. That the filament is hollow is indicated by the appearance of the sporoplasm at the tip only after complete extrusion and the remaining spore contents have left the case.

The only interpretation that seems in accord with the writer's observations is that the extrusion process is one of pressure-induced eversion of a hollow filament followed by its contraction and the rapid passage of the sporoplasm through it. Contraction of the filament after its eversion may be essential to ejection of the sporoplasm as that process often did not occur in filaments extruded onto the glass surface. Their adherence to that surface may have interfered with such contraction.

Stempell (1909) conceived the filament as coiled about the long axis of the spore and surrounded by a girdle-shaped sporoplasm. A different interpretation seems more in accord with the above observations. Although the spore contents are difficult to observe in such small refractile objects, it seems that the filament forms a

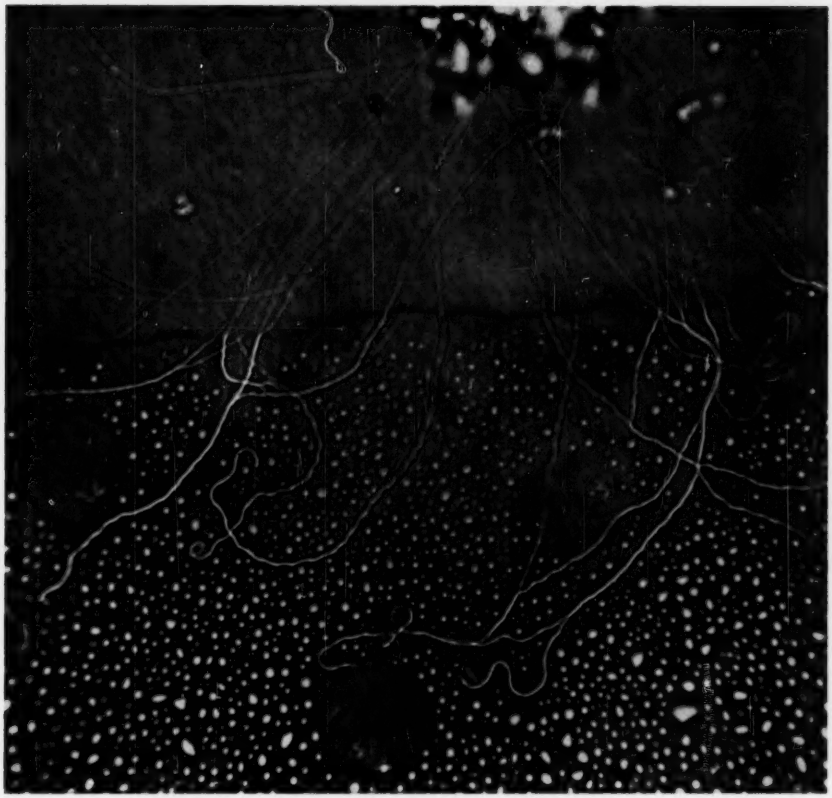

FIGURE 1. Filament extrusion in hanging film preparation. From mass of spores (upper center) filaments extend from fluid onto glass (below) with droplets of condensed moisture. Large drop at lower center with a sporoplasm at end of filament. A partly extruded filament just below the spore mass is spiral in form.

coil in the peripheral rather than the axial region of the spore and that the sporoplasm lies at its apex near the point at which the filament emerges.

The writer's interpretation of spore structure is shown schematically in pl. I, fig. 22. The filament, attached to a hollow plug at the apex of the spore, extends about two-thirds the distance to its base and then peripherally to form a coil which extends toward the apex of the spore in a decreasing number of turns as indicated by the thickness of the coil observed in optical section (pl. I, fig. 13). The filament terminates in the apical end of the spore as a thin sack containing fluid and the sporoplasm which adheres to the side of the sack near its opening into the filament. This saccate tip of the filament evidently everts to form the blister upon which the sporoplasm is seen after complete filament extrusion. The vacuole occupying the basal third of the mature spore may be represented in immature spores by a much smaller region with a marked affinity for neutral red and believed to be within the club-shaped structure seen in such spores stained with hematoxylin (pl. I, fig. 8).

SUMMARY

Nosema sp. develops primarily in the fat body, causing darkening of the larvae and often death before or during the pupal period. Stages in the life history are described and the spore is interpreted as having the polar filament coiled in the peripheral region with its free end expanded to form a sac containing the sporoplasm.

Observations on filament extrusion beyond suspended films onto glass indicate that pressure within the spore causes the eversion of a hollow filament through which the sporoplasm passes to appear at the tip. Penetrating ability of the filament and its firm attachment to the spore case were demonstrated by feeding spores to ciliates.

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EXPLANATION OF PLATE I

FIGURE 1. Meront.
FIGURE 2. Same, dividing by binary fission.
FIGURE 3. Same, at maximum size.
FIGURE 4. Sporont.
FIGURE 5. Sporoblast.
FIGURE 6. Same, with strands forming.
FIGURE 7. Same, with single central strand.
FIGURE 8. Same, with club-like structure.
FIGURE 9. Spore case forming.
FIGURE 10. Spore case complete.
FIGURE 11. Mature spore in saline.
FIGURE 12. Same, imbibing fluid and showing internal structure.
FIGURE 13. Same, extrusion beginning.
FIGURE 14. Same, filament extruding.
FIGURE 15. Same, sporoplasm remaining after filament extrusion.
FIGURE 16. Same, extrusion complete, spore empty.
FIGURE 17. Sporoplasm just emerged.
FIGURE 18. Blister formed at tip of filament.
FIGURE 19. Sporoplasm after blister bursts.
FIGURE 20. Spore showing faulty extrusion as a result of age or coverglass pressure.
FIGURE 21. Spore case with unusual sculpturing.
FIGURE 22. Spore structure schematically represented. CF, coiled filament; EF, everting filament; SC, spore case; SP, sporoplasm; VA, vacuole.

SUGGESTIONS TO PROSPECTIVE AUTHORS

III. ANNOUNCEMENT OF TWO-COLUMN FORMAT FOR THE JOURNAL OF PARASITOLOGY

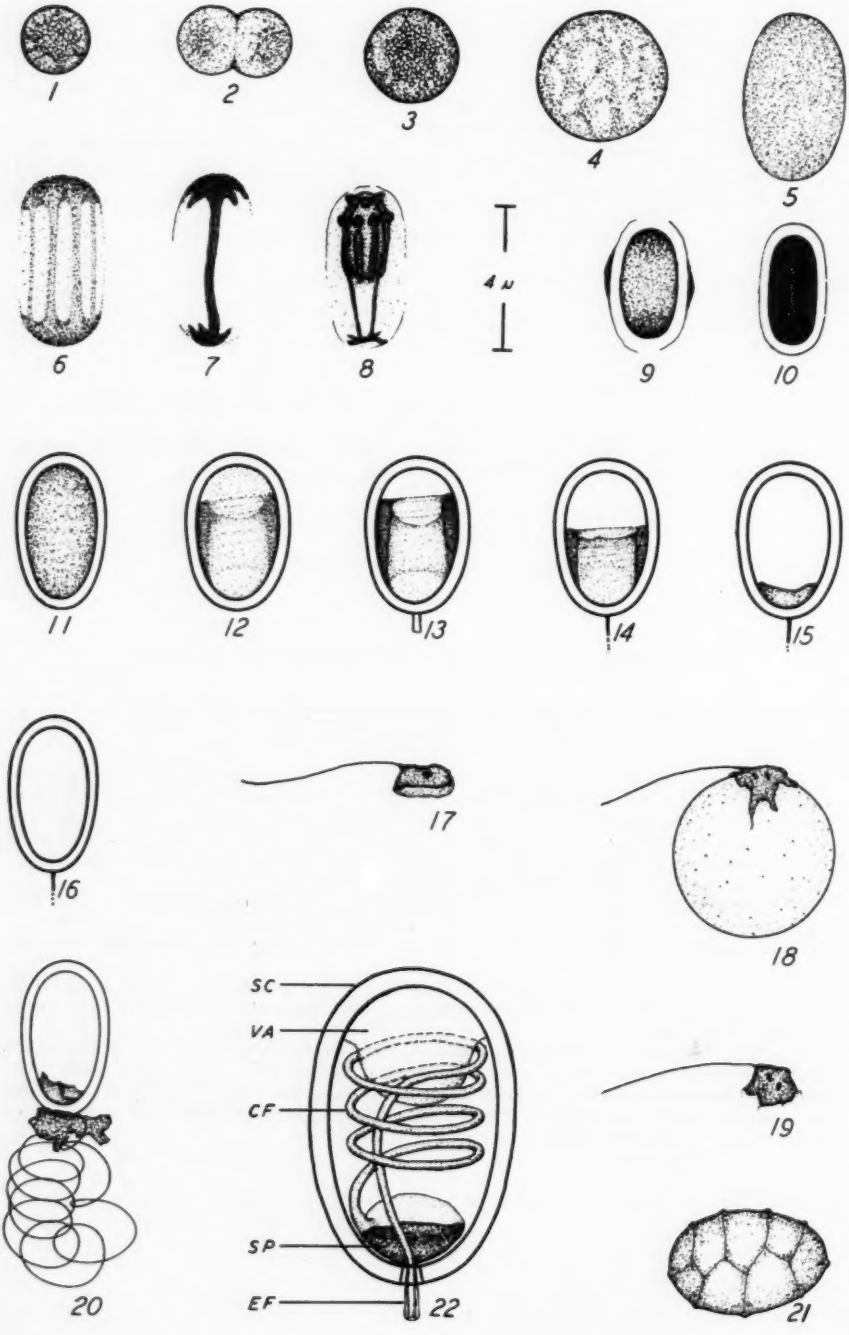
A poll by the Editor of the associate editors, assistant editors, members of the Council, and other officers of the Society indicates that the change to a two-column format for the Journal of Parasitology is strongly favored. The change in format of regular papers will be put into effect as soon as possible; the present format of Research Notes will be continued. There are on hand, however, a number of galleys with the one-column arrangement which must be published before the issues come to consist entirely of two-column papers.

The new columns will be 16 picas (2.667 inches) wide and 51 picas (8.5 inches) long. The 1 pica separation of the columns will make the type page 33 picas (5.5 inches) by 51 picas (8.5 inches). The present overall trim size of the pages will be retained, but the margins will obviously be somewhat narrower.

The present plan is to convert to 9 on 11 point type for what is presently set in 10 on 12 point, but in the DeVinne style. Eight on 9 and 6 on 6 types will be continued for what is presently set in them.

Figures (text), whenever possible, should be prepared so that they will not lose essential detail when reduced to the 16-pica width. The same holds for tables. Wider figures and tables should be submitted only when absolutely necessary. Ordinarily, problems will lie in the width of figures and tables rather than in their breadth. No changes are contemplated in the size of plates.—ELERY R. BECKER, *Editor.*

PLATE I



TWO NEW MICROSPORIDIAN PARASITES OF THE WINTER MOTH, *OPEROPHTERA BRUMATA* (L.)

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In 1956 Professor G. C. Varley (University of Oxford) made available to me for study a number of 5th-stage larvae of the winter moth *Operophtera brumata* (L.) the labial silk glands of which were opaque white and distended. The appearance of the diseased glands was strongly suggestive of a microsporidian infection and this was confirmed on microscopic examination by the presence of large numbers of oval spores from which single polar filaments could be extruded by mechanical pressure.

The stages of development of the parasite within the larvae have been observed and an account of the life cycle is given below. The parasite belongs to the family Nosematidae, characterised by the possession of oval spores and to the genus *Plistophora* in which the sporont develops into a variable number of spores ranging in this case from about 10 to 100 or more.

Of fifty-seven 5th-stage larvae and 24 pupae of *O. brumata,* also collected at Oxford and examined in the summer of 1959, two larvae were found infected with the same parasite and one larva and one pupa with yet another microsporidian species, also in the salivary glands. This species belongs to the genus *Nosema.*

Krieg (1956) described a microsporidian from *Cheimatobia brumata* L. (= *Operophtera brumata* (L.)), this being a species of *Thelohania* parasitic in the fat body and hemocoele of the host. The spores measured 5.00 to 5.75 microns by 2.5 to 3.25 microns, and the polar filament 40 to 80 microns. He named the species *Telohania cheimatobiae.* The species of *Plistophora* described below is easily distinguished from it by the characteristic grouping of the spores and from other species of *Plistophora* by its very small spores (2.3 to 3 microns by 1.0 to 1.5 microns) and its relatively large vegetative phases. It is considered a new species for which the name *Plistophora operophterae* n. sp. is proposed. The *Nosema* sp. was found once in a newly formed pupa and once in a larva ready to pupate. In the pupa there were only the resistant spores of the parasite. In the larva a few cells harbored vegetative phases, but it was not possible to see all the developmental stages. No polar filaments were seen in spite of the numerous methods used to extrude them. In other respects the appearance of the spores was so typically microsporidian that I did not hesitate to place the parasite within the order. That the spores are produced singly from a sporont establishes it in the genus *Nosema* and the size and characters of the spores are used to assign it to a new species, *Nosema operophterae* n. sp.

MATERIALS AND METHODS

The fifth instar larvae and pupae, obtained from Professor Varley, were collected in the Oxford area. Two hundred and thirty 5th-stage larvae collected at Sunninghill, Berkshire, were free of infection.

Smears of the tissues were either dry-fixed and stained with Giemsa or wet-fixed in Carnoy's

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fluid or formol saline and stained by the Giemsa-colophonium technique (Shortt and Cooper 1948). The slight enlargement of the stages resulting from the smearing technique made these preparations suitable for examination of nuclear and cytoplasmic details. Pieces of tissue for sectioning were fixed in Schaudinn's fluid, Carnoy's fluid or formol-saline and stained with Giemsa-colophonium or Heidenhain's iron-haematoxylin. Sections were used only to determine the grouping of the spores and the tissues infected, as the shrinkage of the small parasites, consequent on the treatment required for sectioning, rendered the parasites too small to distinguish details. For smears and sections the Carnoy-Giemsa-colophonium technique was the most satisfactory. The Feulgen reaction, using the technique suggested by Jírovec (1936), was tried on smears and sections, but satisfactory positive results were not obtained, probably because of the small size of the parasites. Fresh spores were stained with toluidene blue but there was no trace of metachromatic material.

Because it was not possible to see the nuclei in fresh preparations, measurements of the successive vegetative stages were made on stained preparations and for comparative purposes measurements of spores were also made in this manner. It is, however, important to remember that the vegetative phases were enlarged by the smearing technique, whereas the thick-walled spores were not. This will, in part, account for the apparent large size of the schizonts and sporoblasts in relation to the size of the spores. Spores were also measured fresh, in 0.75 percent saline solution. In the case of *P. operophterae*, filament extrusion was effected by mechanical pressure.

All measurements are in microns.

Plistophora operophterae n. sp.

The primary site of infection by *P. operophterae* is the labial silk glands, where isolated cells or whole glands may be attacked. Heavily infected larvae may also harbor the parasite in large numbers in the epithelial cells of the gut, the Malpighian tubules, muscles, and fat body.

The youngest stages observed were spherical or nearly spherical, uninucleate trophozoites measuring 2.7 to 3.5 in diameter or 4.5 by 2.5. The cytoplasm stains bright blue with Giemsa's stain. The large nucleus, which occupies nearly half the diameter of the body in smaller specimens, is irregular in outline, stains deep pink and is often associated with a vacuole (fig. 1). As a rule these trophozoites increase in size until they measure approximately 4 in diameter and then undergo nuclear division. Nuclear division is almost certainly amitotic: the nucleus becomes dumbell-shaped, the two halves take up a position at an acute angle to one another and finally separate. Binucleate schizonts (4.5 diameter) and tetranucleate schizonts (5 to 6 diameter) are produced, both being roughly spherical. The cytoplasm becomes coarsely vacuolated and the nuclei are smaller in relation to the size of the body (figs. 2 and 3). At the tetranucleate stage cytoplasmic cleavage begins and uninucleate daughter schizonts are separated (fig. 4). These are ovoid bodies measuring an average of 3.25 by 2.5. The nucleus is about one-third of the diameter of the schizont and is surrounded by a vacuole. In figure 4 two of the daughter schizonts have already begun a further nuclear division. Sometimes the schizont does not divide at the 4-nucleate stage but undergoes further nuclear division so that schizonts with up to 16 nuclei are produced. The larger schizonts with 12 or more nuclei are rare and measure up to 8 by 15. A schizont with 16 nuclei is illustrated (fig. 5) also a group of 13 daughter schizonts derived from division of a large schizont (fig. 6).

Among schizonts in various stages of development, groups of daughter schizonts were occasionally found which were smaller and more elongate than those described above, each possessing two nuclei. The average measurements were 3.1 by 1.4. The cytoplasm was the same deep blue of the normal schizonts but was little if at all vacuolated. The nuclei were compact and small, stained deep red, and were in close

proximity to one another (figs. 7 and 8). Other bodies with similar staining reactions were found separately, some with paired nuclei and others with a single nucleus (fig. 9). The cytoplasm was drawn out in many of these into a long tail at each end; these processes were curled round on the main body of the cytoplasm. These bodies represent a pre-sporogony stage and will be termed pre-sporonts.

The majority of workers on Microsporidia have accepted the view that a sexual fusion is manifested during the life cycle of the Microsporidia by a process of autogamy. Further it appears that in the majority of species this is initiated at the end of schizogony by division of the daughter schizont's nucleus without division of cytoplasm so that binucleate bodies are formed. The completion of the process by fusion of the two nuclei appears to take place at different phases in different species. In monosporous species, where a sporont gives rise to a single spore, it is possible for the fusion to take place at any stage prior to schizogony in a new host. My own observations on *Nosema locustae* (Dissanaike and Canning, 1957) show that the sporoplasm is binucleate throughout its development and after emergence from the spore. If, then, fusion of the nuclei takes place it must do so in the new host before schizogony begins. Dissanaike (1957) showed that in *N. helminthorum* the nucleus divided at the end of schizogony and fused either immediately or late in the development of the spore. Mature spores were typically uninucleate. In polysporous species where the sporont by nuclear and cytoplasmic division gives rise to several sporoblasts and later spores, if nuclear division at the end of schizogony gives rise to binucleate sporonts, the autogamous fusion must take place immediately to allow the zygotic nucleus to undergo division to produce the sporoblast nuclei. However, several workers have reported binucleate spores in polysporous species. Zwölfer (1926) reported occasional binucleate spores in *Plistophora blochmanni* though most spores were uninucleate, and Jirovec (1936) using Feulgen reagent showed that many polysporous species had two nuclei in the sporoplasm. In these cases either autogamy takes place at the end of schizogony and there is a further nuclear division in the sporoblasts to produce binucleate sporoplasms, or autogamy is delayed until after the sporoblasts are produced. Possibly both methods are obtained.

In *P. operophterae* there is little doubt that the complete autogamous process takes place at the end of schizogony. In the final cycle of schizogony the schizonts are small with non-vacuolated cytoplasm. Small daughter schizonts are produced in which the nuclei undergo division; the two daughter nuclei remain in close contact with one another. These binucleate bodies are pre-sporonts. Almost immediately nuclear fusion takes place to produce the sporonts.

At the beginning of sporogony the sporont is an ovoid or elongate body with dense blue, non-vacuolated cytoplasm and a single compact nucleus. It undergoes division to produce the sporoblasts. As nuclear division proceeds the cytoplasm changes in character so that it now stains pink instead of blue with Giemsa's stain. Small vacuoles are occasionally seen, but the cytoplasm remains relatively non-vacuolated compared with that of the schizont, and the nuclei remain smaller. (figs. 10 and 11). It is possible, then, to distinguish schizonts and sporonts by the color of the cytoplasm, size and (sometimes) number of nuclei, and state of vacuolation of cytoplasm. Cytoplasmic division can take place after about the 10-nucleate stage. One group of seven sporoblasts was observed but it was not certain that it was complete. As the cytoplasm collects round the individual nuclei to form

the ovoid sporoblasts the membrane forming the outer wall of the sporont separates and remains as a thin covering to the sporoblasts. In smear preparations this membrane tends to break down. It is likely that the group of seven sporoblasts observed were separated from a larger group. The upper limit of numbers of sporoblasts is more difficult to fix but is probably over 100. The sporoblasts (fig. 12) are ovoid, measure an average of 3.1 by 1.8, and have compact cytoplasm and a single compact nucleus which may be situated anywhere in the cytoplasm. The sporoblasts become surrounded by a thick wall and become spores (fig. 14).

Groups of spherical or slightly ovoid bodies (fig. 13) with a thick wall and deeply-staining, irregular contents were found frequently among groups of spores. It is not possible to say with any certainty what part, if any, these play in the life cycle of the parasite. The presence of the thick wall suggests that they represent an intermediate stage between the sporonts and the obviously recognizable spores, but it is difficult to explain why the contents should be distorted and so deeply staining when the spores which presumably have a thicker wall, less permeable to fixatives and stains, should fix and stain so well. They are of similar size (2.1 by 1.7) but contrasting shape to the spores.

The spores measured under the same conditions as the sporoblasts are smaller, 2.3 to 3 by 1.0 to 1.5, partly because the sporoblasts without the thick wall become smeared out on the slide while the thick wall of the spore prevents this happening to any great extent, but partly also because the cytoplasm of the sporoblast condenses when it forms the spore membrane. Inside the spore some of the cytoplasm separates off as the uninucleate sporoplasm and takes up a position lining the inner surface of the spore membrane. The remainder becomes the polar filament which occupies the central space in the spore. Many spores, both fresh and stained, show a small vacuole at one end (fig. 16).

The polar filament measures only about 25 microns and is exceedingly fine, being difficult to see even when stained. When extruded under mechanical pressure the sporoplasm can be seen at the tip of filament (fig. 15). The sporoplasm is irregular in outline, sometimes amoeboid, often elongate and sometimes rounded. The nucleus is variable, elongate, round, dumbell-shaped or annular.

When sporogony is complete the thin membrane covering the groups of spores breaks down so that spores from neighboring sporonts merge together. It is often difficult at a late stage of infection to observe the grouping of the spores characteristic of the genus *Plistophora.*

Few of the previously described species of *Plistophora* have spores as small as those of *Plistophora operophterae. Plistophora thienemanni* (Debaisieux, 1928) Weiser 1943 from *Chironomus* sp. has very small spores, 2 by 1.5, of a shape differing from that of the spores of the present species as they are broader and more nearly spherical. *Plistophora destruens* Delphy, 1916, parasitic in the muscles of *Mugil auratus,* has slightly larger spores, 2.5 to 3.5 by 1.5 to 2.5, differing also in the shape (pyriform) and the length of the polar filament (10 to 12 times the length of the spore). The brief description of *Plistophora acerinae* Vaney and Conte, 1901, from the fish *Acerina cernua* forms an inadequate basis from which to discuss its relationships with other species. The spores measuring 3 by 2 are slightly broader than those of *P. operophterae.* The spores of *Plistophora collessi* Laird 1959, measuring 2.2 to 2.9 by 1.2 to 1.7, most nearly correspond to the size of the spores from *Operophtera brumata.* However, the filament is shorter (15) and the mature

spores have both an anterior and a posterior vacuole. Further the sporoblasts are formed as discrete masses within the sporont, whereas the whole sporont divides into sporoblasts in *P. operophterae.*

Nosema operophterae n. sp.

Nosema operophterae was found once in a 5th-stage larva and once in a newly formed pupa. Only the silk glands were parasitized and these not heavily. Isolated groups of two or three cells were attacked and these were packed with spores, but adjacent cells were normal. There were very few parasitic forms other than spores present. A few binucleate schizonts were seen (fig. 17) which measured 3 to 5 by 1.5 to 2. The cytoplasm stained intensely blue with Giemsa's stain and the nuclei stained deep pink. Spherical, uninucleate bodies were present in small numbers (fig. 18). These measured 2.5 to 3 in diameter and had dense cytoplasm forming a peripheral ring surrounding a vacuole. The nucleus was situated at the edge of the vacuole. No doubt these are the products of the last division of the schizonts and later become sporonts. The sporonts are ovoid (fig. 19) and measure 2.7 to 4 by 1.3 to 2. The cytoplasm is coarsely vacuolated and stains deep blue. The large nucleus is situated at or near one pole. The spores (fig. 20) are less deeply staining, probably because the thick spore membrane is less pervious to stains. The majority of spores measured in smears, fall into a size range of 2.5 to 3.5 by 1.0 to 1.3, i.e. they are about the same width as the spores of *P. operophterae* but longer. After the spore membrane is formed the cytoplasm recedes from one end of the spore forming a vacuole; the nucleus, coming to lie at the edge of the vacuole, divides into two. The mature spore has two nuclei lying close to one another at the edge of the vacuole. When it was found that the polar filament could not be extruded by mechanical pressure a number of other recommended methods were tried, viz, drying and immersion of spores in distilled water (Gibbs 1953), hanging drop preparations in distilled water or 0.75 per cent saline solution, hydrogen peroxide, iodine solution, ether, and hydrochloric acid (Kudo 1924). None had any effect on the spores, and it was not possible to try other reagents owing to lack of material. The appearance of the infected glands, the structure of the mature spores (excepting only the lack of evidence for a polar filament) and the development of the spores from the sporonts are so typically microsporidian that, in spite of being unable to show the filament characteristic of the Microsporidia, I do not hesitate to place the parasite in this order. A small number of spores (less than 1 percent) are much longer than the normal type, many about twice the length, but have the same width. The maximum length observed was 7.7, but the majority of the large spores fell into a size range of 5 to 7 by 1.0 to 1.5. The cytoplasm is not vacuolated. There are two nuclei sometimes lying near one another at the center of the spore, otherwise lying widely separated (fig. 21). The most likely explanation of their presence is that they develop from two incompletely separated sporonts. A binucleate schizont would normally divide to give two uninucleate sporonts which in turn would become spores, but if after elongation of the binucleate schizont to undergo cytoplasmic division the spore wall was formed before the division could take place, the long spores thus formed would represent double spores. The nuclei do not divide again. The lack of vacuoles indicates that development is arrested after spore wall formation.

Of the many species of *Nosema* recorded parasitic in Lepidoptera nearly all

have spores larger than those of *N. operophterae*. Its spores can be compared in size only with those of *N. vanillae* β Lutz and Splendore, 1903, and since only the spores of this species were studied by the authors its generic status has not been confirmed. It can be distinguished from *N. operophterae* by the shape of the spores, (one end slightly broader than the other in *N. vanillae* and strictly cylindrical in *N. operophterae*).

PATHOGENICITY AND TRANSMISSION

As *N. operophterae* was found on only two occasions, little can be said of its pathogenic effects. The parasite was found only in the silk glands and here the infection was restricted to isolated cells. No information can be given regarding the effect of the parasite on the length of life of the host. One of the two infected insects had pupated. The infected cells were considerably hypertrophied, about twice the size of the normal cells, but the nuclei were unaffected. The parasites were located towards the hemocoele boundary of the cells, leaving an area of unattacked cytoplasm towards the lumen of the gland. Thomson (1959) noted that spores of *Perezia disstriae* parasitic in the silk glands and mid-gut epithelium of *Malacosoma disstria* were found in the silk secretion. This is not so in *O. brumata* parasitized with *N. operophterae* probably because of the area of unattacked cytoplasm bordering the lumen of the gland.

P. operophterae attacks primarily the silk glands of its host; in many cases infection is limited to this site. The sporoplasm enters the cells and undergoes a period of rapid division. The cell soon becomes heavily infected with schizonts and some enter a phase of sporogony. In this case, the onset of sporogony is not initiated by overcrowding in the host cell. Some cells harboring few parasites have several groups of spores, whereas cells virtually packed with parasites have schizogony continuing alongside sporogony. Gradually more and more cells along the length of the gland are attacked. As with *N. operophterae* the parasites become concentrated on the hemocoele side of the cell displacing the nucleus towards the lumen side and leaving an area of unaffected cytoplasm bordering the lumen of the gland. No spores were found in the lumen. Later the infection may spread to cells of the gut, Malpighian tubules, fat body, and muscles. Because of the position of the parasites in the salivary gland cells, it seems likely that schizonts and spores are released by breakdown of the host cells into the hemocoele where they can attack directly the Malpighian tubules, fat body, and muscles. That the gut epithelial cells are also attacked via the hemocoele is indicated, for the parasites always attack first the side of the epithelial cells towards the hemocoele, later spreading throughout the cell. All parasitized cells are hypertrophied. The gut and Malpighian tubules, like the silk glands, are sites for heavy infection. Cell boundaries break down leaving whole areas packed with spores interspersed with host cell nuclei. The muscles are less heavily infected, though considerable damage is done in patches. The fat body is lightly infected, parasitized cells often being difficult to find.

The larvae seem little affected by the parasite. Their external appearance and activity are normal and 5th-instar larvae though heavily infected are able to pupate. It is doubtful, however, if infected insects ever emerge from the cocoons. Many hundreds of adult moths collected in the same area as the infected larvae have been examined but no trace has been found of the parasite.

The eggs of the winter moth are laid during the colder months of the year and can be found on the bark of many deciduous trees in cracks. The larvae hatch in spring, feed on developing flower and leaf buds and undergo four molts to produce the 5th-instar larvae. In summer these drop off the trees, make their way underground and pupate. The adults emerge in winter and come above ground: the wingless female climbs up the tree trunk, is fertilized by the winged male and, having laid her eggs mostly on the upper branches of the tree, dies shortly after. The male lives a few weeks.

If parasitized larvae can pupate, but most die within the cocoons, the infective stage of the parasite would remain buried below ground; the new generation of larvae hatching on the trees would not come into contact with the infection.

Obviously some infective spores will be passed out during the life of the larva, particularly those parasitic in cells of the gut and Malpighian tubules, and these spores, being on the trees, will serve as a source of infection to the larvae the following spring. Whether other methods of transmission, such as transovarial, are possible is the subject of further investigation.

SUMMARY

Two new Microsporidia, *Plistophora operophterae* n. sp. and *Nosema operophterae* n. sp., are described from *Operophtera brumata* (L.) The latter species is found only in the salivary glands, whereas the former attacks cells of the salivary glands, gut, fat body, muscles, and Malpighian tubules.

Schizonts of *P. operophterae* stained with Giemsa's stain have deep blue cytoplasm, contain large vacuoles and large, irregular deep pink nuclei numbering up to 16. Uninucleate schizonts measure 3.25 by 2.5. At the end of schizogony presporonts are produced which measure 3.1 by 1.4. These undergo autogamy and give rise to sporonts. The cytoplasm of the sporonts stains pink, is homogeneous, and the nuclei are small and compact. From 10 to 100 sporoblasts are produced measuring 3.1 by 1.8, and these develop into uninucleate spores 2.3 by 1.5.

Only binucleate and uninucleate schizonts of *N. operophterae* were seen. The uninucleate schizonts, 2.5 to 3 in diameter, give rise to ovoid sporonts 2.7 to 4 by 1.3 to 2, which in turn give rise to spores 2.5 to 3.5 by 1.0 to 1.3. The spores, at first uninucleate, undergo autogamy so that the mature spore is also uninucleate.

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EXPLANATION OF PLATE I

FIGURES 1 to 16. *Plistophora operophterae.*
FIGURE 1. Uninucleate schizonts.
FIGURE 2. Binucleate schizonts.
FIGURE 3. Tetranucleate schizonts.
FIGURE 4. Division of tetranucleate schizont.
FIGURE 5. Multinucleate schizont.
FIGURE 6. Products of division of multinucleate schizont.
FIGURES 7 and 8. Groups of binucleate pre-sporonts.
FIGURE 9. Late pre-sporonts, some binucleate, others after nuclei have fused.
FIGURE 10. Tetranucleate sporont.
FIGURE 11. Multinucleate sporont; one sporoblast separated.
FIGURE 12. Group of sporoblasts.
FIGURE 13. Thick-walled bodies, possibly intermediate between sporoblasts and spores.
FIGURE 14. Group of spores to show similar size but contrasting shape to figure 13.
FIGURE 15. Emerged sporoplasms, some with filaments.
FIGURE 16. Mature spores.
FIGURES 17 to 21. *Nosema operophterae.*
FIGURE 17. Binucleate schizonts.
FIGURE 18. Uninucleate schizonts.
FIGURE 19. Sporonts.
FIGURE 20. Development of spores.
FIGURE 21. Double spores.

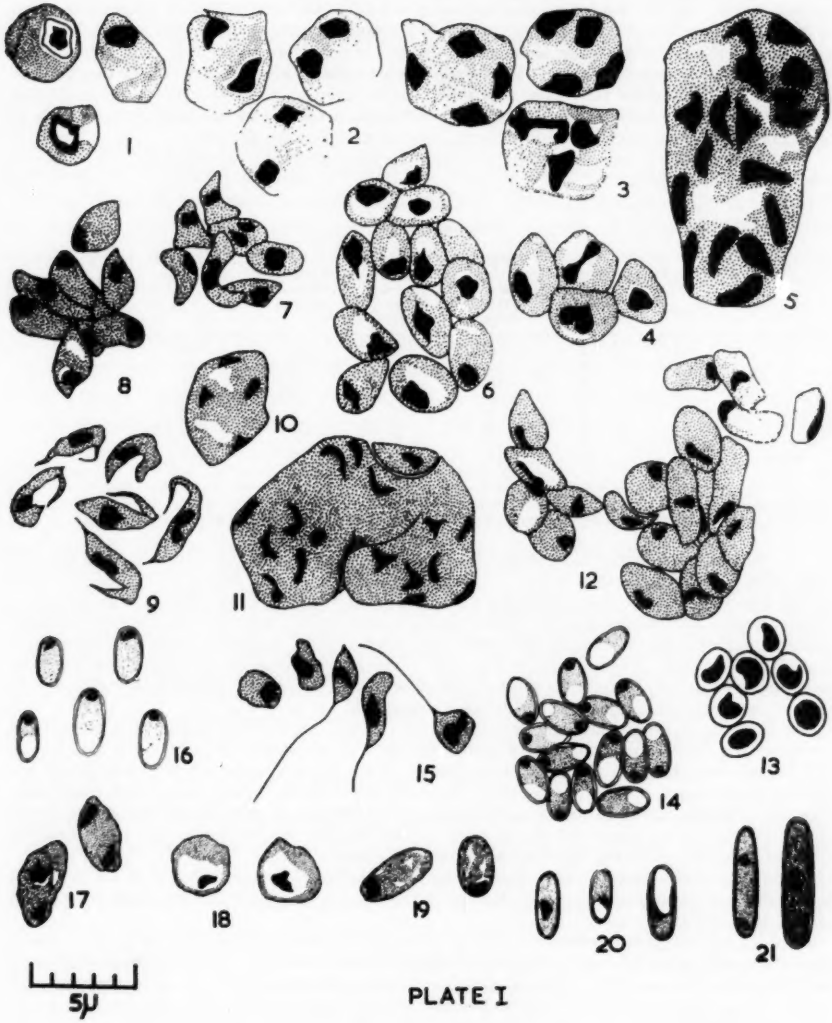

PLATE I

RESEARCH NOTE

WHITE RATS INFECTED WITH *LITOMOSOIDES CARINII* SUITABLE FOR ROUTINE SCREENING OF FILARICIDE COMPOUNDS.

In two papers (1959, Zschr. Tropenmed. Parasit. **10:** 70–77; *ibid.* **10:** 385–401) the author proved that the experimental infection of white rats with filarial worms of the cotton rat (*Litomosoides carinii*) can be used for routine screening of macro- and microfilaricide compounds. Out of 289 laboratory rats, 204 (71 percent) could be infected with these filariid parasites. The rats were killed 160 days at the latest after being infected; in nearly all the animals microfilariae and living mature worms were found. The blood was examined twice a week for larvae, first 50 days after infection of the rats. The first microfilariae could be detected in the peripheral blood about 60 days after infection. The growth rate of filariae in white rats was nearly the same as in cotton rats (maximal length of adult male 3.2 cm; of adult female, 11.6 cm).

In the meantime a new group of 99 animals has been infected experimentally with *Litomosoides carinii* (see table).

Group no.	Total	Number of rats				Total number of filarial worms	Total length of dead filarial worms (cm)
		With living filarial worms only	With living and dead filarial worms	With dead filarial worms only	Without filarial worms		
1	14	4	0	0	10	18	0
2	15	6	9	0	0	403	64
3	15	9	5	1	0	201	26
4	16	6	2	0	8	45	10
5	14	4	9	0	1	304	62
6	14	6	8	0	0	396	59
7	11	6	5	0	0	146	62
Total	99	41	38	1	19	1513	283 (5.8 percent)*

* The datum given in percentage refers to the share of dead filarial worms in relation to the total number of filariid parasites. Fragments of 3-cm length are calculated as one worm.

These rats were autopsied in seven experimental groups between the 71st and the 130th day after being infected (average, 91 days). In five groups only one rat out of 69 proved free from filariid parasites. In the two remaining groups, a great number of the animals was free from these parasites, because many of the mites (*Bdellonyssus bacoti*) used for transmission had died. Each rat harbored on the average 19 worms, and often, besides, a small number of fragments of dead filarial worms. These fragments do not interfere with routine examination as they are surrounded by a large number of pleuraendothel cells and, therefore, can be distinguished very easily from worms recently killed by filaricide compounds.

It must be stressed that only one out of 80 infected rats, when examined post mortem, harbored only dead filarial worms. Microfilariae could be detected in the peripheral blood in all cases where both sexes of the worm were found. Only one rat harboring a single female and another harboring two male worms were free from microfilariae. Only once, after mild infection and with low concentration of microfilariae in the blood, the microfilariae transiently could not be found. In all other rats the number of microfilariae in the peripheral blood remained constant or increased during the duration of the experiment.

Based on these results, which must be considered even more positive than those described in the above-mentioned papers, the following method has proved its value for routine screening of possible filaricide agents.

Albino-rats weighing between 30 and 40 g are placed twice, at intervals of 1 week, into a cage containing infected tropical rat mites (*Bdellonyssus bacoti*). After 60 days, 0.05 cc of blood is taken from the tail of the rat, put into a centrifuge tube, and 5 cc of distilled water is added. Then this mixture of blood and water is centrifugalized for 5 minutes at 3000 r.p.m. After centrifugalization, the fluid is decanted until a small residue remains which is filled into the chamber of a Zschucke counting cell. The microfilariae are counted using a magnifying power of 1 : 60. If the result is positive, the compound to be checked is applied to the rat. After 1 or 2 days a new check of microfilariae is carried out. After a week another compound is applied, etc. In the experimental series we use the rats for about 120 days after infection.—KLAUS ROHDE, *Zool. Dept., University of Malaya in Kŭala Lŭmpŭr, Pantai Valley, Kŭala Lŭmpŭr, Malaya.*

OBSERVATIONS ON THE HOST HEMOGLOBIN DURING *PLASMODIUM LOPHURAE* INFECTIONS IN CHICKS*

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Studies of protein alterations of the avian host during the malarial infection have been restricted exclusively to investigations of the serum and plasma and to quantitative decreases in the hemoglobin content of the erythrocyte (Groman, 1951). Since the malarial parasite is an obligate, intracellular parasite and utilizes the hemoglobin of the erythrocyte as a nutritive source, it is possible that the quality of the hemoglobin may exert some influence on the course of the infection. Chickens typically show two electrophoretically distinct hemoglobins (Johnson and Dunlap, 1955); thus, the possibility exists that the two hemoglobins could be differentially synthesized in vivo and absence or depletion of one of the hemoglobins during the course of the malarial infection might adversely affect the growth and reproduction of the parasite. In addition, electrophoretic analysis might also reveal an extracellular proteolytic action of the parasite on the hemoglobin.

MATERIALS AND METHODS

To explore the above three possibilities, blood was withdrawn from chicks under one month of age previous to infection and during the course of infection with *Plasmodium lophurae* at the peak and after the peak of parasitemia, and heparinized. Erythrocytes were washed in a buffer solution without albumin (Trager, 1954), lysed by suspending them in one-half their volume of 0.15 percent saponin in the same buffer and incubated at 39 C for 15 minutes. At the end of this period, a volume of buffer sufficient to bring the total volume to 1 ml was added to the lysate. The diluted lysate was centrifuged in the cold (4 C) at 20,000 *g* for 20 minutes. The supernatant was decanted and its hemoglobin concentration adjusted with distilled water to 2 to 3 g per 100 ml, using the acid hematin method described in Hawk, Oser and Summerson (1954) as the estimator of hemoglobin concentration. Samples of 0.005 ml hemoglobin plus 0.005 ml of 7.5 percent bovine serum albumin (Difco), added to serve as a mobility reference, were analyzed by paper electrophoresis as previously described from this laboratory (Sherman and Hull, 1960).

RESULTS, DISCUSSION, AND CONCLUSIONS

Analysis of the chicken hemoglobins by paper electrophoresis at three points, viz, previous to, at the peak of, and after the peak of infection, shows that "Hyline" chicken hemoglobin is separable into two entities which stain with bromphenol blue and are of distinct quantity and quality (fig. 1). Arbitrarily, the faster moving electrophoretic species was designated alpha-hemoglobin while the slower one was termed the beta-hemoglobin. The alpha component usually comprises about 25 percent, and the beta about 75 percent of the total amount of hemoglobin. Although there is somewhat more variability in the relative quantity of peak and post-peak period hemoglobins than in the hemoglobins of the uninfected chick, such variations do not show

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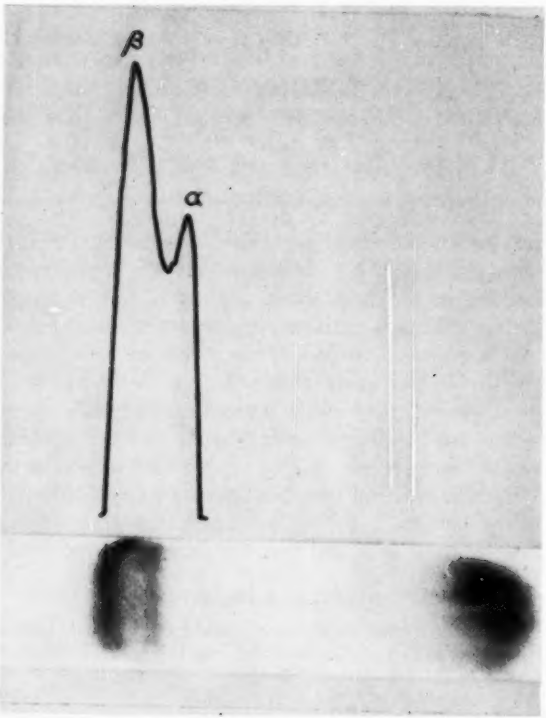

FIGURE 1. Representative strip and densitometric pattern of the hemoglobins of the chick when analyzed by paper electrophoresis. The protein band at the anodal (right) end of the strip represents the 7.5 percent bovine serum albumin added to serve as a mobility reference.

statistical significance when compared to the uninfected hemoglobin values.

It was found that an electrophoretic run of at least 12 hours was required to effect adequate separation of the hemoglobins in the apparatus used (horizontal free strip; see Moinat & Tuller, 1957), but this period allowed the bovine serum albumin to migrate to the buffer bath rests. Since mobility rate in the free strip is not equal to the rate in the supported strip, accurate mobility calculations were not possible.

These studies indicate that (1) there is no detectable degradation of the erythrocyte hemoglobin by the parasite outside of the parasite body, since no new electrophoretic entity appears in response to the presence of the parasite in the erythrocyte, and (2) the host synthesizes its normal hemoglobins in much the same proportions as in the uninfected birds in spite of increased anemia and reticulocytosis.

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RESEARCH NOTE

A MODIFIED FILTRATION AND AERATION UNIT FOR EXPERIMENTAL SNAIL AQUARIA.

In working with schistosomes or other trematodes which require aquatic molluscs as intermediate hosts it is usually necessary to maintain small aquaria for groups of exposed or shedding snails. Most laboratories provide some means of aeration of the water, frequently in the form of stone air-breakers connected to an aquarium pump or compressed air line. This system provides no filtration and produces fine water droplets, which may contain cercariae, which in the case of human schistosomes constitute a potential hazard to the laboratory worker.

Under the gravel filtration devices for larger aquaria (5 and 10 gallon) are available from aquarium supply houses. Aquaria of these sizes are frequently used to maintain stock breeding colonies of snails. We installed such commercially available filtration and aeration devices in our stock colonies and noted a definite improvement. This observation led to the adaptation of the system to our experimental aquaria. For maintenance of these smaller groups of exposed and shedding *Australorbis glabratus* we are using pyrex jars, 6 by 6 inches. The filter plate is constructed from one-eighth inch plexiglass cut in the form of a circle to fit inside the pyrex jar (fig. 1). This circular plate is perforated by one-eighth inch holes on three-fourths inch centers and is supported from the bottom by three one-half inch square blocks of one-fourth inch plexiglass. A one-inch square of one-fourth inch plexiglass is cemented to the top of the filter plate at the periphery. A hole is drilled through both the block and the filter plate and threaded to receive the threaded end of a one-half inch O.D. plexiglass tube 4 inches in length. A one-fourth inch O.D. plexiglass tube 4.5 inches long is cemented inside the half-inch tube so that the end is one-half inch from the threaded end of the larger tube and projects 1 inch above the top of it. The one-fourth inch tube is connected to the air line by flexible plastic tubing. Upon installation in the aquarium the circular filter plate is covered with a layer of glass wool and aquarium gravel or sand placed on top of the glass wool. Aquatic plants are planted in the gravel in the usual manner. Air bubbles escape from the small tube rise in the larger tube causing the water to be lifted from beneath the filter plate resulting in a circulation of the water through the gravel and glass wool providing effective filtration as well as aeration. Activated charcoal may be mixed with the gravel or a layer of charcoal placed between the gravel and glass wool. This system does not eliminate the droplet problem but does minimize it when compared with the use of stone air-brakers which produced many small bubbles. Aquaria equipped with the filtration and aeration units herein described remain clear and usable for many months. (Supported by research grant E-2500 from the U. S. Public Health Service)—DONALD V. MOORE, *Department of Microbiology, University of Texas Southwestern Medical School, Dallas, Texas.*

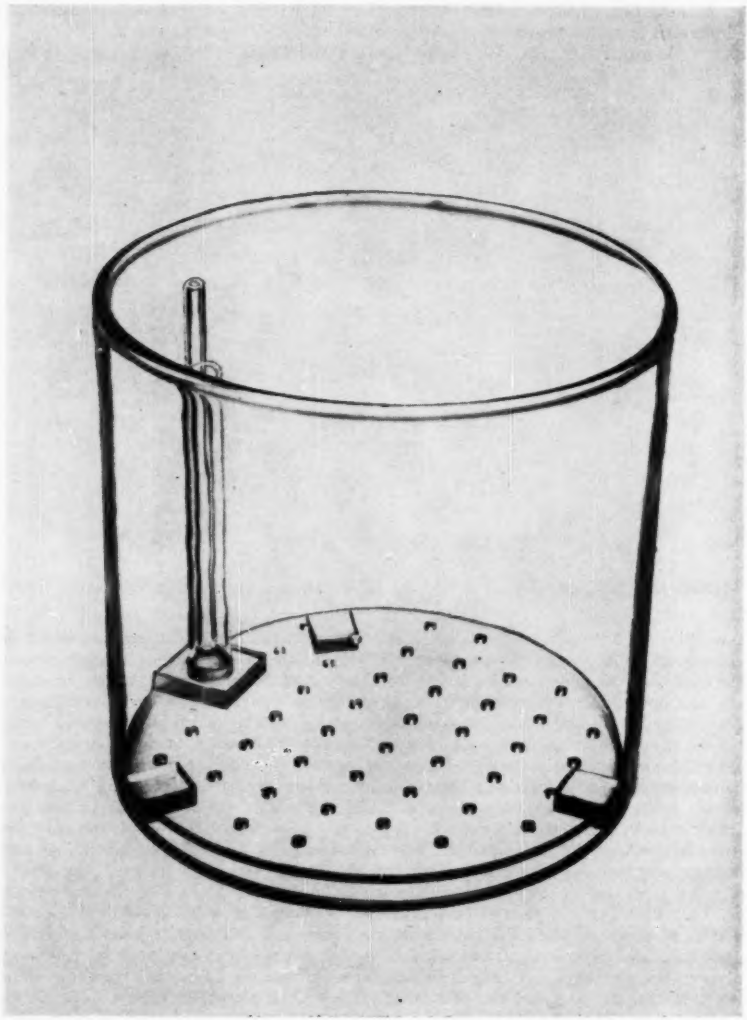

FIGURE 1. Modified filtration and aeration unit in an experimental snail aquarium.

NATURE AND POSSIBLE SIGNIFICANCE OF THE PIGMENT IN FASCIOLOIDIASIS

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Infections of the large North American liver fluke, *Fascioloides magna* (Bassi, 1875) Ward, 1917, are characterized by black pigmentation of the host tissues. The pigmentation occurs in infections of various Cervidae, the common hosts, and to a greater extent in infections of the less common and less tolerant hosts, sheep and cattle. In infected sheep the pigment frequently becomes distributed throughout the parenchyma of the liver; the omentum becomes heavily mottled over its entire surface; the lymph glands become almost black, and patches of pigment may be found in other organs, especially the diaphram and lungs.

It has been reasonably assumed that the pigment associated with fascioloidiasis is simply the dispersed vomitus of the flukes (Swales, 1935). This hypothesis is supported by the fact that in the present investigation the solubility properties of the pigment extracted from lymph tissue were found to be exactly like those of pigment from the intestine of the flukes.

The pigmentation of fascioloidiasis has been referred to in the literature as "melanosis"; the black fluid bathing the flukes has been called "melanotic fluid" (Olson, 1949); and the pigment itself has been called a "melanoid pigment" (Price, 1953). However noncommittal these terms may be, they are liable to give the impression that the pigment is a melanin compound. Nowhere in the literature reviewed is there a positive statement concerning the nature of this pigment. If *Fascioloides* feeds on blood, which has not in fact been established, it would seem more likely that the pigment would be a breakdown product of hemoglobin. As part of a general study of fascioloidiasis (Campbell, 1957) an attempt was made to determine the nature of the pigment.

MATERIALS AND METHODS

The general distribution of the pigment was observed in infected sheep. Pigmentation was also studied in fresh livers and adjacent lymph glands of infected deer (*Odocoileus virginianus*, Boddaert) and cattle, but whole carcasses of these hosts were not examined.

The pigment, though soluble in alkali, was not readily extracted in alkali, and was insoluble in most solvents. It was, however, found to be quickly and easily extracted from both tissue and fluke by liquefied phenol. Such extracts, when dried in an oven, yielded a glossy black solid residue. Because of the gross unsuitability of obtaining pigment in this way (vide infra), a digestive solution consisting of 1 g pepsin and 0.5 ml hydrochloric acid in 100 ml water was used to digest the fluke tissue and leave the indigestible gut content behind. Before using the digestive solution, the uterine area of the fluke was removed, because the eggs are not digestible. Flukes which had been frozen when fresh were thawed and incubated in the solution at 38C. This procedure was very effective; the body of the fluke quickly disintegrated and after a period of 1 to 1½ hours nothing solid remained but numerous black particles of intestinal content. This pigment was collected by repeated sedimentations and washings in distilled water. When dried by evaporation, the pigment appeared under the microscope to consist of amorphous particles, though when viewed without magnification it had an almost crystalline appearance. Although the digestive

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solution would split any contaminating hemoglobin molecules, the large quantity of washed pigment particles obtainable by this method essentially obviated the danger of getting a spurious indication of hematin as a result of such splitting. Pigment was not extracted from liver tissue by this procedure. Absorption spectra of the pigment and its hemochromogen were recorded on a Cary spectrophotometer.

RESULTS

1. *Phenolic extracts.* The solid residue obtained by extraction in liquefied phenol was found to be soluble in dilute alkali (0.2 percent sodium hydroxide). It was not soluble in boiling water, but dissolved readily in boiling concentrated sulfuric or hydrochloric acid. Over a 24-hour period it also dissolved slowly in concentrated hydrochloric acid at 31C. It was insoluble in chloroform and in hot 95 percent ethyl alcohol, and was practically insoluble in a 3:8 mixture of pyridine and chloroform. The dried residue was virtually insoluble in liquefied phenol and the color of the material was only slightly reduced by hydrogen peroxide.

These results, considered as a whole, are inconclusive; they include properties characteristic of, and properties alien to, both melanin and hematin compounds. The insolubility of the material in phenol suggests that the pigment had been altered by extraction and storage in phenol. Furthermore, phenolic extracts are unsuitable for the detection of heme compounds in a source where unaltered hemoglobin might have been present, since phenol will split any contaminating hemoglobin and give a misleading indication of hematin (Deegan and Maegraith, 1956).

It was clear, therefore, that another method of isolating the pigment had to be found. The use of strongly basic solutions is not advisable because of the likelihood of altering the pigment. Heme compounds are iron-porphyrin complexes, and tests for iron have been suggested for distinguishing heme from melanin (Sawada, et al, 1956). However, melanins in nature often do in fact contain iron (Laxer and Whewell, 1954) and, therefore, precise quantitative tests would be necessary if the iron content were to be a sound criterion.

2. *Pigment obtained by digestion.* Pigment obtained from the intestinal branches of flukes by pepsin digestion, was soluble in liquefied phenol, dilute alkali, sulfuric alcohol, concentrated formic acid, and was very slightly soluble in acetone. It was insoluble in 1.0 N hydrochloric acid at 31C, benzene, ether, chloroform, pyridine, glycerine, 3:8 mixture of pyridine and chloroform, ferric chloride, glacial acetic acid, and 0.1 M borate buffer. This pigment could be precipitated by the addition of hydrochloric acid to an alkaline solution. The precipitate was reddish-black and could be collected by centrifuging. Neither the original pigment nor the precipitate lost its form or color when heated in a furnace at 600C. The addition of hydrogen peroxide (30 percent) or sodium hypochlorite (Chlorox) to an alkaline solution of the pigment caused an immediate decolorization.

The above properties are in accordance with those generally listed as characteristic of melanin; viz, soluble in alkali, insoluble in acids and organic solvents, bleached by oxidizing agents. When these tests were performed on a laboratory sample of hemin, however, it was found that most of these properties appertain also to at least 1 heme compound. Stephenson (1947) reported somewhat similar properties for the gut content of *Fasciola hepatica* and stated that, according to Lison (1936), those properties denoted either melanin or the malarial pigment (shown by Deegan and Maegraith (1956) to be combined hematin).

Since solubility and bleaching tests are thus manifestly ill-suited to even the broadest determination of the fascioloidiasis pigment, it was resolved to employ

spectrophotometric methods to compare the absorption characteristics of the pigment with those of a known heme compound.

Absorption records were first made of very dilute alkaline solutions of both pigments, using visible light. The absorption in the 300 to 450 millimicron portion of the spectrum is shown in figure 1. This record shows a great absorption peak at

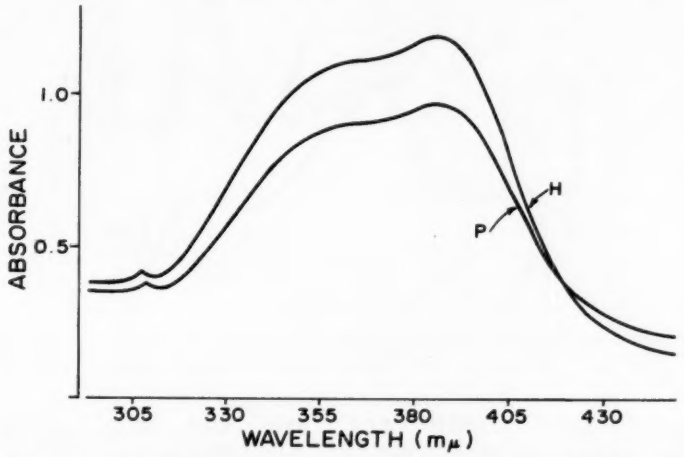

FIGURE 1. Absorption of alkaline solutions of fascioloidiasis pigment (P) and hemin (H) in visible light; spectrophotometric recording between approximately 300 and 450 millimicron wavelengths. Note absorption peak below 400 millimicrons, characteristic of compounds with intact porphyrin ring.

just under 400 millimicrons, a peak known as the Soret band, and characteristic of compounds having an intact porphyrin ring. Melanins are reported to have no absorption characteristics in visible light (Fox, 1953); this curve would thus eliminate the possibility that the pigment of *Fascioloides* is melanin. Figure 1, furthermore, shows a striking agreement between the curves of the fluke pigment and hemin. A record of the same two solutions was made using ultraviolet light, and again both pigments presented similar records. The absorptions of more concentrated solutions of the fluke pigment and hemin, recorded in the 450 to 650 millimicron portion of the visible spectrum are shown in figure 2. These curves are in essential agreement, making it extremely probable that the fluke pigment is an iron-porphyrin.

For further confirmation, hemochromogens of both the *Fascioloides* pigment and the laboratory sample of hemin were prepared. Hemochromogens are derivatives of hemoglobin which are formed in the presence of a base, a reducing agent, and a source of nitrogen, and which are renowned for their characteristic absorption spectra. In the present work the alkaline solutions of the fluke pigment and the hemin were reduced with a mixture of glucose and base, and the source of nitrogen was pyridine. The resultant pyridine-hemochromogens were pale reddish liquids, and their absorption characteristics in visible light were compared by means of the recording spectrophotometer. The absorption record obtained for the 450 to 675 millimicron portion of the spectrum are shown in figure 3. In this record the absorption curve of the *Fascioloides* pigment hemochromogen is indicative of a heme compound,

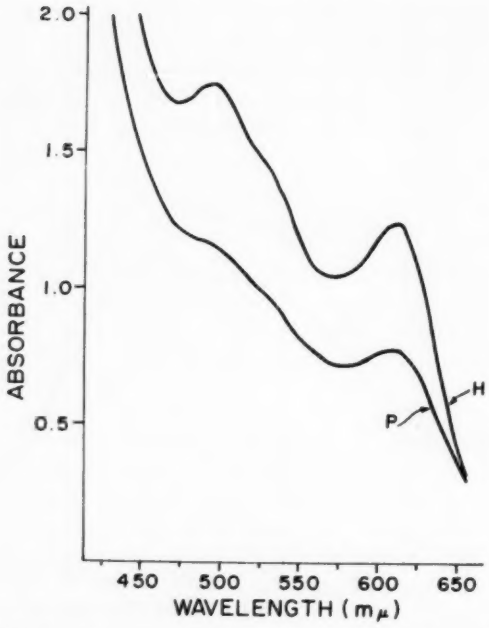

FIGURE 2. Absorption of more concentrated solutions of the fascioloidiasis pigment (P) and hemin (H) in visible light; spectrophotometric recording for 425 to 650 millimicron wavelengths.

and besides shows a remarkable coincidence with the curve of the hemochromogen prepared from the laboratory sample of hemin.

These findings demonstrate that the *Fascioloides* pigment and the hemin sample are chemically similar. The source of the laboratory hemin sample could not be ascertained, but presumably the term hemin refers to chlorohemin ($C_{34}H_{32}O_4N_4FeCl$). When dissolved in excess alkali this compound would become hematin ($C_{34}H_{32}O_4N_4FeOH$) (Lemberg and Legge, 1949). Similarly if the *Fascioloides* pigment was originally present as hemin it also would be changed to the hydroxide form when dissolved in sodium hydroxide for spectrophotometric examination. Thus it is not possible, on the basis of this limited study, to state the nature of the pigment more specifically than that it is a heme compound.

DISCUSSION

It has been observed that deer livers containing immature migrant flukes are invariably mottled with pigment, whereas livers containing encapsulated flukes, and no migrants, may or may not be pigmented. The capsules themselves always contain pigment, either in a thin fluid surrounding the flukes, or in a clay-like material filling old capsules in which the flukes have died. Apparently, then, tissue pigmentation is associated with the presence of non-encapsulated flukes; after encapsulation has occurred the pigment is gradually removed from the parenchyma, and the pigment formed thereafter is either eliminated through the bile duct openings of capsules or accumulated within capsules. Thus an infection in a liver exhibiting cap-

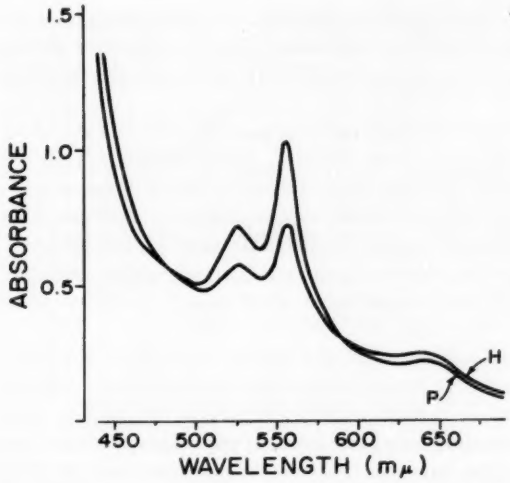

FIGURE 3. Absorption of pyridine hemochromogens of the fascioloidiasis pigment (P) and of hemin (H); spectrophotometric recording for 450 to 675 millimicron wavelengths.

sules and pigmentation (but no migrant flukes) would be a younger infection than in a liver containing fluke capsules, but no parenchymal pigmentation or migrant flukes.

In the course of other studies on fascioloidiasis the author concluded that the remarkable differential tolerance of different host species was not due to differences in the degree of mechanical destruction of liver tissue, resulting from encapsulation or non-encapsulation of the flukes (Campbell, 1957). It has been suggested instead that the encapsulation observed in deer and cattle, but only rarely in sheep, affords protection by enclosing and, in the case of deer, eliminating, toxic parasite products which would otherwise be distributed throughout the liver, and indeed throughout the body. Such toxic products could act on organs other than the liver, or could act upon the liver itself, thereby inhibiting some vital metabolic process. The deleterious parasite product could conceivably be the pigment, especially since hematin is known to inhibit succinoxidase activity (Keilin and Hartree, 1947). Attempts to discover the inhibition of specific functions of intermediary metabolism in infected sheep included comparison of the rates of oxidization of sodium succinate in infected and non-infected sheep liver. In a small number of tests no significant difference could be demonstrated.

Moderate pigmentation occurs in immature infections in deer. Therefore, if the elimination of the pigment is to account for the tolerance of deer as opposed to the intolerance of sheep, the pigment would be expected to exert its ill effects only when present in excess. Unfortunately most of the few sheep available for tissue respiration studies had only light immature infections. If the pigment is free hematin the lack of inhibition of succinoxidase may have been due to an insufficient amount of hematin in the tissue studied. On the other hand, one sheep was virtually moribund when killed and yet showed no succinoxidase inhibition in spite of pigmenta-

tion which was rather more than moderate. It is tentatively concluded that, although the pigment of fascioloidiasis may conceivably be deleterious above a certain threshold level, it does not exercise its effect by inhibiting succinoxidase activity of the liver.

The liver of mice infected with schistosomiasis is discolored by a dark pigment generally regarded as hematin (Fairley, 1920; Meleney, et al, 1952). Daugherty (1955) reported that the liver of mice infected with *S. mansoni* had a reduced ability to oxidize succinic acid. It would seem desirable to establish definitely whether or not the schistosomiasis pigment is chemically uncombined hematin, in which case it might account for the reported decrease in succinoxidase activity, or whether it is coupled with a nitrogenous entity, in which case it would not (Keilin and Hartree, 1947).

According to Swales (1936), the wall of the *F. magna* capsule in infected deer livers is provided with blood capillaries. Of more than 400 capsules examined grossly by the author, a small number exhibited a thin broken wall, tinged with red as a result of numerous petechial hemorrhages. This condition may have been due to the feeding of the flukes or to incomplete or abnormal capsule formation. The great majority of capsules, however, had a thick white fibrous wall with no visible trace of surface blood supply or hemorrhage. *F. magna* does not possess obvious penetrating equipment, and has not been observed with its anterior tip embedded in the capsule wall; it is therefore difficult to understand how such encapsulated flukes could obtain blood. Although such synthesis does not occur in mammals, it is tempting to speculate that *F. magna* might conceivably synthesize heme compounds from bile pigments, perhaps using iron conserved from its early migrating phase.

SUMMARY

The nature and possible significance of the black pigmentation associated with infections of *Fascioloides magna* have been discussed. It has been concluded that the black material filling the branched intestinal crura of *F. magna* consists chiefly of an iron-porphyrin, and that the pigmentation associated with fascioloidiasis represents the deposition of this heme compound in the host tissues.

ACKNOWLEDGMENTS

This investigation was carried out in the laboratory of Dr. A. C. Todd, University of Wisconsin. The spectrophotometric recordings of the pigment and its hemochromogen were made by Dr. Julius Adler, then of the Enzyme Institute, Madison, Wisconsin, to whom grateful acknowledgment is hereby made.

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RESEARCH NOTE

EXPERIMENTAL TRANSMISSION OF RUMINANT NEMATODES OF THE GENERA *COOPERIA, OSTERTAGIA,* AND *HAEMONCHUS* TO LABORATORY RABBITS.

Information on the infectivity of the genera *Cooperia, Ostertagia,* and *Haemonchus* to small laboratory mammals is limited. Recently, *Cooperia punctata* was successfully transmitted from cattle to rabbits and was reported not to be particularly adaptable to that abnormal host (Alicata, 1958, J. Parasit. **44** (supp.) : 30). Infective larvae of *Haemonchus contortus* were transmitted to guinea pigs and recovered in the lungs in advanced stages of development (Ransom and Foster, 1920, U. S. Dept. Agr. Bull. 817). Natural infections of *H. contortus* have been reported in ground squirrels (Sassuchin and Tiflow, 1933, Ztschr. Parasitenk. **5:** 254). No reports were found of *Ostertagia* spp. from ruminants being established in laboratory mammals.

During an investigation of the host-parasite relation of the ovine nematode *Trichostrongylus colubriformis* in rabbits, concurrent infections of *Cooperia curticei* and *O. circumcincta* were found at necropsy. These newly observed host-parasite relations and data on the bovine species of *Cooperia* and *H. contortus* from sheep are reported here.

Six to 10-week-old male New Zealand White rabbits were used as hosts. Standard parasitological techniques were used to culture infective larvae, inoculate rabbits, make fecal egg counts, digest host tissues, and evaluate the infections.

The experimental infections of *C. curticei* are recorded in table I. Characteristic cooperia

TABLE I. *Experimental infections of* Cooperia curticei *in rabbits.*

Experiment	Days post-inoculation to necropsy	Number of rabbits		Total number of worms recovered	Male to female ratio
		Inoculated	Infected		
A	30	1	1	45	0.80
	36	2	2	90	0.78
	68	1	1	65	0.63
	211	1	0	0	—
B	90	9	2	5	0.20
C	94	7	2	33	1.10
D	30	3	1	20	all females
	43	3	2	120	0.50
	57	3	3	40	all females
E	127	1	1	12	1.00
	141	1	1	5	0.67
	200	2	0	0	—

eggs were found in the feces; the prepatent period was 18 days. The average male to female ratio was 0.74. In experiment A the species was patent for at least 68 days in the rabbits. One of the two rabbits necropsied at 36 days post-inoculation harbored five gravid female *O. circumcincta*. The rabbit necropsied at 68 days had 5 gravid females and 10 male *O. circumcincta*. In experiment D, the variation in the numbers of rabbits infected and the total number of *C. curticei* recovered may have resulted from the 1 : 40 dilution of intestinal contents used to count a large number of *T. colubriformis* present.

After *C. curticei* was observed to be capable of infecting rabbits, four rabbits each were given 200 infective larvae to determine the degree of infectivity and duration of infection (table I, experiment E). Percent infectivities of 6.25 and 2.5 were found at 127 and 141 days post-inoculation, respectively. Neither of the two rabbits necropsied at 200 days was infected with *C. curticei*.

The results given in table I suggest that a small portion of the *C. curticei* population is capable of normal development in the rabbit host as shown by a normal pre-patent period, establishment of both sexes and by the presence of eggs in the feces of 10 of the inoculated rabbits.

Results of preliminary tests on establishing *C. punctata* in rabbits are given in table II.

TABLE II. *Experimental infections of* Cooperia punctata *in rabbits.*

Rabbit	No. larvae in inoculum	Necropsy		Male to female ratio	Infectivity (percent)
		Days post-inoculation	No. worms recovered		
M-C	not counted	43	73	0.62	—
M-L	1400	17	13	0.63	0.93
M-6	100,000	45	438	0.65	0.44
M-7	100,000	19	690	0.73	0.64
R-1	1380	35	38	0.81	2.80
R-2	1380	35	28	1.15	2.00

Rabbits M-C and M-L were given an inoculum containing several genera of infective larvae cultured from the feces of a naturally infected steer and an experimentally infected lamb, respectively. *Cooperia punctata* was established with low infectivity in the rabbits. Rabbit M-C passed 57 eggs per gram of feces 17 days post-inoculation. This count gradually decreased to four, 40 days after inoculation. A single male of *C. oncophora* was recovered from rabbit M-C. Rabbits M-6 and M-7 were given a large inoculum containing the single species, *C. punctata*, to determine the degree of infectivity and to establish a large number of worms in the host.

To investigate the results of the second generation *C. punctata* infection in rabbits, known numbers of infective larvae of this species cultured from eggs in the feces of M-C were given to rabbit R-1 and R-2. The results of these few first and second generation infections suggested that the infectivity may have more than doubled after one generation passage. This work indicates an area of future study on the adaptation of an initially poorly adapted species to an abnormal host through successive passages.

Seventy-five rabbits each receiving as many as 100,000 ensheathed infective larvae of *Haemonchus contortus* with a mixed inoculum of *T. colubriformis* contained no *Haemonchus* at necropsy. Of two rabbits each given 100,000 artificially exsheathed *Haemonchus* larvae, one necropsied 16 days post-inoculation had eight 5th-stage females and five 5th-stage males, while the rabbit necropsied 5 days later had an adult male and female *H. contortus*.

Contribution No. 292, Department of Zoology, Kansas State University, Manhattan and Zoology Series No. 331.—IRWIN B. WOOD, *Parasitic Chemotherapy Section, Agricultural Division, American Cyanamid Co., Pearl River, N. Y.*, AND M. F. HANSEN, *Department of Zoology, Kansas State University, Manhattan.*

TYPES AND SOURCES OF PIGMENTS IN CERTAIN SPECIES OF LARVAL TREMATODES*

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Several parasitic organisms belonging to different animal phyla are known to exhibit brilliant coloration in a diversity of patterns and colors. This is particularly true of many helminths. Although casual references to pigmentation have been made by various authors in taxonomic and morphological literature, to the present author's knowledge no organized work has been undertaken to study the chemical nature and origin of pigments found in larval and adult helminths. Several kinds of pigments like haemoglobin and its derivatives, cytochromes, flavins, protoporphyrin, and coproporphyrin, have been reported from parasitic worms (Brand, 1952; Fox, 1953). Carotenoid pigments are known to occur in polyclad turbellarians (Francotte, 1898); in parasitic infusorians like *Spirophrya, Polyspira,* and *Gymnodinioides* (Chatton et al, 1926) and in a crustacean parasite, *Sacculina* (Lenel, 1954). The only record of the occurrence of a carotenoid pigment in a helminth has been made by Van Cleave and Rausch (1950) who reported to have obtained characteristic color reaction for carotene in an acanthocephalan, *Arhythmorhynchus comptus.*

The present report is concerned with the chemical nature and origin of pigments found in sporocysts, rediae, and cercariae of a few species of trematodes. The various larval stages are colored yellow, orange, brown, blue-green, green, and so on. In order to trace the sources of pigments in these parasites, it was necessary to study the pigments of the marine snail, *Cerithidea californica* Haldeman, in which these larvae develop, and of the algae upon which the snails feed. Efforts have been made to correlate the host-parasite relationship of pigments by way of the food-chain cycle.

MATERIALS AND METHODS

Materials used for the present investigation include: three species of green algae, *Ulva* sp., *Chaetomorpha torta,* and *Enteromorpha clathrata;* the red alga, *Hypnea johnstonii;* the snail host, *Cerithidea californica;* and the trematode larvae of the following types: *Cercaria buchanani* (orange sporocysts), *Catatropis johnstoni* (orange redia), fin-tailed echinostome (pale orange rediae), large pigmented echinostome (green cercariae; rediae with pigmented gut), Y-bladder cercaria (yellow sporocysts), *Cloacitrema michiganensis* (green rediae; rediae with pigmented gut), *Parorchis acanthus* (pale orange rediae), small echinostome (orange rediae), and small xiphidiocercaria (light yellow sporocysts). The algae and the snails were collected from the mud flats of Upper Newport Bay, California, during the period extending from 1955–58.

Extraction and chromatographic separation of various algal pigments were carried out according to the methods reported by the following workers with necessary modifications: Strain (1942) for chlorophyll a and b, and carotenoid pigments; Manning and Strain (1943) for chlorophyll d; and Haxo et al (1955) for phycobilins.

The assorted snail tissues such as the digestive glands, mantle, and visceral mass were separately lyophilized and ground in an ordinary mortar before the extraction of pigments. The larval types were also carefully sorted out free of snail tissues, and kept under refrigeration until

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sufficient amounts of materials were obtained. Lyophilization and subsequent grinding of the larval materials were also done as in the case of snail tissues. The methods outlined by Fox and Pantin (1941) were followed with slight modifications for the extraction and analyses of carotenoid pigments from the parasitic larvae and the snail.

In all cases, an adsorption column was prepared in a cylindrical glass tube measuring 20 cm by 12 mm. Preparation, setting up of the adsorption column, development of the chromatogram, separation of the colored zones, etc., were carried out for carotenoid pigments as described by Karrer and Jucker (1950). The following adsorbents were used for the separation of the various pigments: Powdered sugar (C and H Confectioner's, dried in an oven at about 85 C for about 8 hours) for chlorophylls; tricalcium phosphate for phycobilins; activated alumina for carotenes, and calcium carbonate or zinc carbonate for xanthophylls. Activated alumina was found very effective for the separation of both epiphasic and hypophasic carotenoid pigments. Among several developing solvents used, petroleum ether (b.p. 50 to 70 C) with 1–5 percent methanol proved most satisfactory for chlorophyll and carotenoid pigments.

Chromatographically separated pigment fractions were eluted in appropriate solvents like petroleum ether, hexane, or methanol, to determine the absorption spectra in ultra violet and visible light using a Beckman spectrophotometer. Identification of the pigments has been made by spectrophotometric absorption analyses, color reactions, partition tests, solubility, fluorescence, and chromatographic behavior. Recorded measurements are for wavelengths, expressed in millimicrons.

The works of the following authors, besides those cited in the course of this paper, have also been very helpful in identifying the various pigments: Atkins and Jenkins (1953); Beadle and Zscheile (1942); Carter et al (1939); Goodwin (1953); Green (1957); Harris and Zscheile (1943); Rabinowitch (1951); Strain (1951, 1952); and Vevers and Millott (1957).

MORPHOLOGY OF LARVAL PIGMENTATION

Fresh materials were used for the morphological studies on account of the solubility of pigments in organic solvents which precludes the use of paraffin sections. The tissues of the infected snails such as the digestive gland, mantle, etc. appeared brilliantly colored orange, yellow, brown, or blue-green owing to the presence of pigmented parasites. It was rather easy to determine whether or not a given snail was infected with pigmented larval forms by merely observing the tissues since they showed a characteristic and distinctive coloration.

Some species of rediae are remarkable for the pigmented gut, which appears as a narrow streak or longitudinal band (fig. 1). Microscopic examination of the gut wall indicated the presence of two kinds of oily pigment globules; large yellow and small orange (fig. 2). The gut is full of solid material apparently ingested from the snail host. The entire gut, including its contents as well as its wall, is pigmented. It was possible to observe certain tissue components of the snail host within the gut of the rediae, particularly the "globule-like concretions" (Winkler, 1957), usually present in the digestive glands of certain species of snails.

In species of rediae such as those of the fin-tailed echinostome, *Parorchis acanthus,* and small echinostome, pigmentation extends to the body wall as well (fig. 3). Small specks of orange or yellow pigment are found distributed throughout the body wall. Under higher magnification of the microscope, these pigment specks appear to be more or less star-shaped with a clear space in the center of each (fig. 4). The processes or extensions of these specks seem to be interconnected. Spaces among the scattered pigment specks are free of pigmentation.

Sporocysts like those of the *Cercaria buchanani,* Y-bladder cercaria, and small xiphidiocercaria, have heavily pigmented body walls. The pattern of pigmentation in these larvae is quite characteristic and appears different from that of the rediae. Scattered in the body wall of the sporocysts are several irregularly-shaped pigment specks, each with one or more clear spaces (fig. 5). The intensity of pigmentation

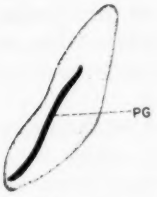

FIG 1

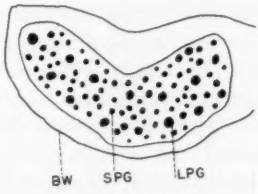

FIG 2

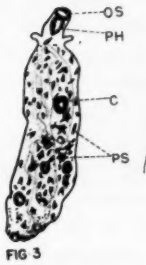

FIG 3

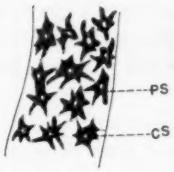

FIG 4

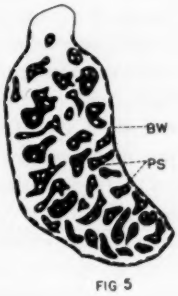

FIG 5

FIGURE 1. A redia with pigmented gut (*Cloacitrema michiganensis*).
FIGURE 2. An enlarged view of portion of the gut wall of the above.
FIGURE 3. A redia of fin-tailed echinostome.
FIGURE 4. A portion of the body wall of the fin-tailed echinostome.
FIGURE 5. An enlarged view of a sporocyst (Y-bladder cercaria).
Abbreviations: BW, body wall; C, cercaria; CS, clear space; LPG, large pigment globules; OS, oral sucker; PG, pigmented gut; PH, pharynx; PS, pigmented spots; SPG, small pigment globules.

of the body wall of the rediae and sporocysts depends upon the number of these specks. In light colored rediae and sporocysts, they are very sparsely distributed. Rediae and sporocysts with heavy and light pigmentation were common in the collection.

In a few instances, cercariae with green coloration have been encountered. Histological examination of the fresh material revealed that the green pigments responsible for this coloration are confined to the cystogenous gland cells with which a mature cercaria is heavily loaded.

OTHER RESULTS

The three green algae have been found to contain chlorophyll a and b, β-carotene, and a xanthophyllic pigment, lutein; the red alga, chlorophyll a and d, β-carotene, lutein, phycoerythrin, and phycocyanin.

The snail host stores both carotenoid and chlorophyllic pigments. β-carotene, lutein, carotenoid acids, zeaxanthin, and chlorophyll derivatives have been identified. β-carotene was the major pigment found in various tissues. Parts of the lutein and one carotenoid acid were found esterified in the digestive gland of the snail. A blue-green residue was left in the aqueous methanolic fraction of the original extracts of the digestive gland and visceral tissues. Even though the chlorophyllic pigments

could be removed by acidified ether, the blue tinge of the solution still remained. Attempts to separate the residual pigment on adsorption columns and extraction by various solvents failed. This bluish residue may represent haemocyanin which is a common respiratory pigment in molluscan body tissues.

The fact that most of the larval pigments dissolved out during the preparation of paraffin sections indicated that these are extremely soluble in organic solvents. Solubility of the pigments was further tested by keeping formalin-fixed sections of the pigmented larvae in various solvents over a period of time. They have been found insoluble in water, formalin, weak acids, and alkalis.

When a few drops of concentrated sulfuric acid were added to refrigerator-dried larval materials, a pale blue color appeared. Pigment samples from different species of larvae in chloroform solution exhibited a greenish-blue color when treated with concentrated sulfuric acid. A positive color reaction was also obtained for the pigment extracts with the Carr-Price test (Glick, 1949). These reactions are characteristic of carotenoid pigments.

A few drops of petroleum ether extract of the pigments from various larvae were placed on a piece of Whatman No. I filter paper and exposed to ultra violet light. The pigment spots in the paper showed green fluorescence.

The pigment extracts from all species of larvae in petroleum ether showed a tendency to undergo decoloration when exposed to atmospheric oxygen.

Absorption spectra of the various larval pigment extracts showed two distinct bands in the blue-violet region. In some cases another band in the red region was also observed. The absorption bands in the blue-violet region were characteristic of carotenoids and one in the red of chlorophyllic pigments.

Thus, the preliminary tests for color reactions, fluorescence, solubility, and spectral properties indicated that the larval pigments belong to carotenoid and porphyrin groups.

No hypophasic pigments could be detected from any of the larval species on partition of the original extracts between 90 percent methanol and petroleum ether systems. Pigments invariably remained epiphasic even after saponification.

Chromatographic separation and subsequent spectrophotometric absorption analyses indicated the presence of β-carotene in the following larval trematodes: *Cercaria buchanani* (sporocysts), *Catatropis johnstoni* (rediae), fin-tailed echinostome (rediae), large pigmented echinostome (cercaria; rediae), Y-bladder cercaria (sporocysts), *Cloacitrema michiganensis* (rediae), small echinostome (rediae), and small xiphidiocercaria (sporocysts). In most cases the color of the band on the alumina column containing the β-carotene was orange and the absorption maxima were at 450 to 454 and 480 to 484 in petroleum ether (fig. 6). However, in the case of large pigmented echinostome rediae, the absorption curve of β-carotene showed three peaks, at 424, 452, and 482 in petroleum ether (fig. 7). Besides β-carotene, Y-bladder sporocysts contained an epiphasic carotenoid compound with single absorption maximum at 452 in petroleum ether, which has been identified as a keto-carotenoid. Two types of rediae, those of the large pigmented echinostome and *Cloacitrema michiganensis*, contained chlorophyll derivatives which showed absorption maxima at 416, 670; 410, 665 respectively in methanol (fig. 8). The pigments extracted from the pale orange rediae of *Parorchis acanthus*, could not be chromatographically separated because of insufficiency of material. However, the absorp-

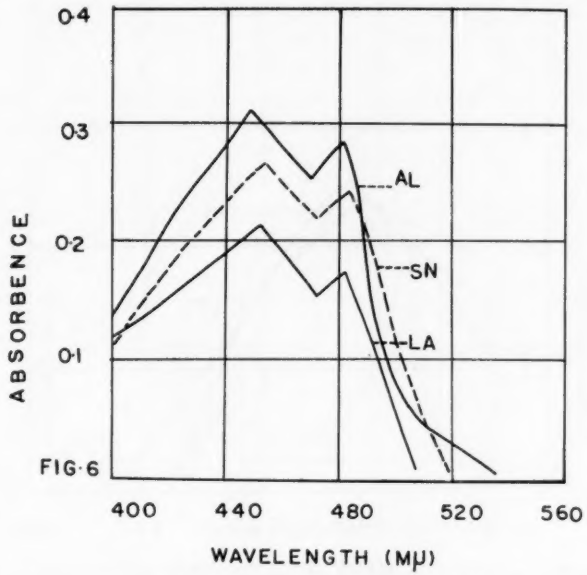

FIGURE 6. Spectral curves of β-carotene from alga, snail, and larva. Solvent: petroleum ether.

tion curve with the maxima at 424, 450, and 660 in methanol indicated that this was a mixture of chlorophyll derivatives and carotenoid pigments.

In all cases during chromatographic separation of the pigment extracts one or two colored bands (khaki or pale yellow or brown) were recovered from the top of the adsorption column. The colored material obtained from these bands did not show any recognizable absorption maxima and hence they could not be identified. They may possibly be some break-down products of chlorophyll or carotenoid pigments since pigments of this nature have very often been found adsorbed at the top of the columns (Fox, 1953).

Pigments from four types of larvae, Y-bladder cercaria (yellow sporocysts), large pigmented echinostome (rediae with pigmented gut), *Cloacitrema michiganensis* (rediae with brown gut), and fin-tailed echinostome (yellow rediae), extracted during the months of June and July 1955, showed only one absorption band with the maxima at 450 to 454 in petroleum ether. Spectral (fig. 7) and other properties indicated that these are carotenoids, probably keto-carotenoids or some pigments derived from β-carotene as isomerization products.

Quantitative difference in pigment contents has been observed among four species of larvae, Y-bladder cercaria (yellow sporocysts), large pigmented echinostome (rediae with pigmented gut), *Cloacitrema* (redia with brown gut), and fin-tailed echinostome (yellow rediae). Yellow sporocysts of Y-bladder cercaria have been found to store more pigments quantitatively than the rest of the species. The quantitative difference in pigment contents is probably correlated with the spatial relationship, intensity of pigmentation, and nutritional state of these organisms.

Tests for flavones, riboflavins, vitamin A, haemoglobin, haematin, bile pigments, haemocyanin and lipofuscines were negative in all the larval types.

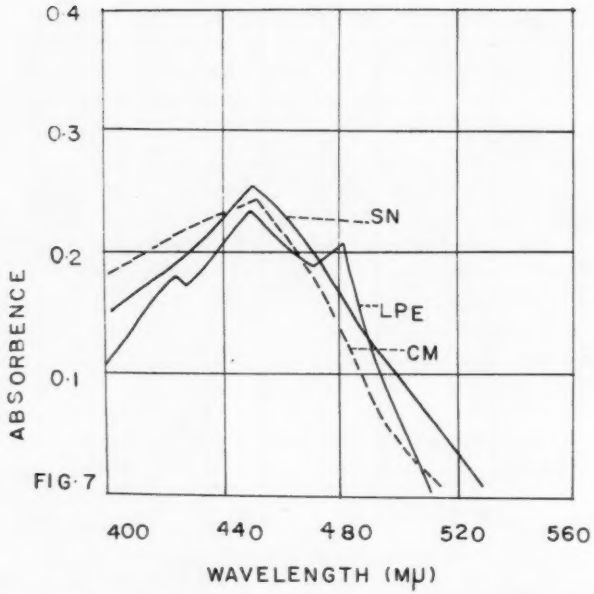

FIGURE 7. Spectral curves of β-carotene from the rediae of large pigmented echinostome; carotenoid pigments with single absorption band from the snail and the larvae. Solvent: petroleum ether.

DISCUSSION

Although carotenoids and chlorophyll derivatives are known to occur in several species of invertebrates, their occurrence in larval trematodes seems not to have been reported before. All species of larval trematodes investigated, except *Parorchis,* revealed the presence of β-carotene. Chlorophyll derivatives and keto-carotenoids have also been recovered from a few larval types.

Since the larval trematodes studied develop in the digestive gland and other tissues of the snail host, *Cerithidea,* the immediate sources of pigments for them are to be sought in the snail itself. They live, feed and grow in these snails. All available evidence, such as those obtained from spectral properties (figs. 6, 7 and 8), color reactions, chromatographic behavior of pigments, observation of ingested snail tissues in the gut of the rediae suggest that the larval trematodes absorb the carotenoid and chlorophyll pigments from the snail host. The nutritional relationship of animal pigmentation in free-living as well as parasitic organisms is well known (Goodwin, 1954; Fox, 1953). Chatton et al (1926) for instance, described how the infusorian parasite, *Spirophrya,* absorbed pigments by digesting the colored eye-material of the copepod, *Idya furcata;* while two other infusorians, *Polyspira* and *Gymnodinioides,* absorbed pigments from the hermit crab, *Eupagurus pridauxii.* The dark brown pigments of several species of polyopisthocotylean monogenetic trematodes have been shown to be haematin derived from host's haemoglobin (Llwellyn, 1954). Literature is full of similar examples illustrating the nutritional aspect of animal pigmentation.

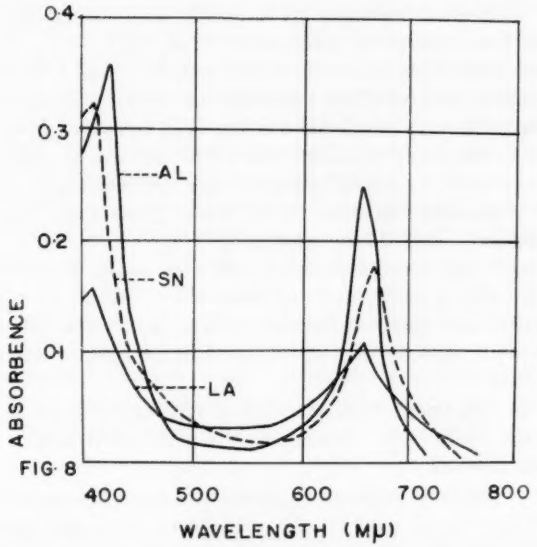

FIGURE 8. Spectral curves of chlorophyll from alga, snail, and larva. Solvent: methanol.

The larval trematodes seem to have absorbed only the carotenes among several carotenoids available in the snail host. Selective absorption of hydrocarbons is rather unique among the invertebrates. In some cases there is a complete exclusion of carotenes as in the California sea mussel, *Mytilus californianus;* this mussel assimilates and stores only the oxygenated xanthophylls (Scheer, 1940). However, preferential accumulation of carotenes has also been noticed in the case of certain echinoids (Fox and Scheer, 1941), a polychaete worm, *Thoracophelia mucronata* (Fox et al, 1948), and certain sponges (Fox, 1953); and in the selective absorption of carotenes larval trematodes may be classified with them.

The spectral and other properties of β-carotene recovered from the algae, snail, and larvae did not show any significant difference (fig. 6), indicating that this pigment is stored unaltered in the tissues of the snail and the larvae. Spectral properties, however, differ in regard to chlorophyll derivatives extracted from the snail and the larvae. The absorption maximum in the violet region of the spectrum of chlorophyll from the algae was at about 432; whereas chlorophyll derivatives from the snail and the larvae showed maxima at 416 and 410 respectively. This shows that the chlorophyll pigment has undergone some kind of metabolic change in the snail and larval tissues. However, the maximum in the violet region of the chlorophyll at 416 or 410 represents the so-called Soret band suggesting that the porphyrin ring in the molecular structure is still intact. The maximum of the chlorophyll derivatives from the snail and the larvae in the red region of the spectrum is normal for chlorophyll a, a fact that suggests the possible derivation of the former pigments from the latter.

β-carotene in the green, large, pigmented echinostome cercaria seems to be conjugated with some protein. No trace of chlorophyll could be detected in the extracts of this cercaria, and the pigment extract in 90 percent methanol and petroleum ether

looked yellow. Several invertebrates are known to contain carotenoid-protein complexes exhibiting blue or purple or green colors (Fox, 1953).

The pigments with single absorption band, extracted from four species of larvae during the months of June and July 1955, may be absorbed as such from the snail since an identical carotenoid compound has also been recovered from the snail (fig. 7). However, it is intriguing to assume that the larvae have absorbed only this pigment from an array of carotenoid pigments, the overwhelming part of which is β-carotene. It is possible that these larvae absorb β-carotene from the snail and store it as metabolically altered keto-carotenoid.

It is remarkable that chlorophyll derivatives were totally absent from the sporocysts. In the absence of mouth and gut, these larvae should be considered as absorbing the pigments and nutrients through the body wall. The failure of sporocysts to absorb chlorophyll pigments may be explained on the basis of selective permeability of the body wall.

There was no indication of the presence of chlorophyll d or phycoerythrin or phycocyanin in the snail's body. Apparently the snail fails to absorb these pigments from the red alga *Hypnea*.

The sources of pigments in the larval trematodes could thus be traced back to the green algae which serve as food for the snail host. The algal pigments undergo various kinds of metabolic changes within the snail, except for the β-carotene and lutein pigments. However, esterification of some of the lutein pigments in the digestive gland of the snail has been observed. Zeaxanthin and carotenoid acids are peculiar to the snail and are to be considered as products formed as a result of metabolic activities of the snail. β-carotene absorbed from the snail seems to be stored in the tissues of the larvae in an unchanged condition. However, in the case of green, large pigmented echinostome cercariae, it exists as a carotenoid-protein complex. Keto-carotenoids are absorbed by the larvae as such from the snail or metabolically derived from some other snail pigment sources, probably β-carotene. The chlorophyll derivatives found in three types of rediae have undergone slight metabolic changes from the condition in which they were absorbed from the snail host.

Biologically, carotenoid and chlorophyll pigments found in the larval trematodes may be of negligible significance. They may be indicators of the degree of unsaturation of trematode fat with an incidental partition coefficient favoring accumulation. It is known, however, that carotenoids may serve to prevent autoxidation of lipids in animal tissues (Verne, 1936a, 1936b) and to play a vital part in phototropism (Millott and Vevers, 1955). Carotenoids and other pigments are sometimes involved in protective and alluring coloration of animals. The brilliantly colored sporocysts of *Leucochloridium* are known to penetrate the tentacles of the snail *Succinea*, rendering them conspicuously colored presumably to lure the birds, their definitive hosts (Wesenberg-Lund, 1931). None of the sporocysts or rediae seems to enter the tentacles of the snail *Cerithidea*, and coloration in the present instance has probably no alluring effect. Protective and alluring coloration, however, should have some survival value to the species; in some parasitic species having complicated life-cycles they may ensure a certain degree of security in finding their hosts or their hosts finding them.

SUMMARY

The pigments of the snail host *Cerithidea* and of four species of algae which serve

as food for the snail have been studied. β-carotene was extracted from three types of sporocysts, six rediae and one cercaria. Only three types of rediae and none of the sporocysts contained chlorophyll derivatives. Besides β-carotene, sporocysts of Y-bladder cercaria were found to contain a keto-carotenoid. Spectral and other properties indicated that the pigments extracted from four species of larvae during the months of June and July 1955 are keto-carotenoids or some pigments derived from β-carotene. All available evidence indicates that the larvae absorb the pigments from the snail host. They seem to absorb only the hydrocarbons from several kinds of carotenoids present in the snail. The larvae appear to store the β-carotene in an unchanged condition. Chlorophyll derivatives in the larvae seem to have undergone some metabolic changes. Tests for flavones, riboflavins, haemoglobin, haematin, bile pigments, haemocyanin, and lipofuscines were negative in all the larval species. Morphological aspects and biological significance of larval pigmentation have been discussed with reference to carotenoid and chlorophyll pigments. It was possible to trace the origin of pigments in the larvae by way of the food-chain cycle involving the algae and the snail host.

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STUDIES ON THE IMMUNOLOGY OF SCHISTOSOMIASIS BY VACCINATION AND PASSIVE TRANSFER

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Immunity against schistosomiasis has been experimentally demonstrated in laboratory animals and many studies have been made on related serological reactions. However, the relationships of these reactions to immunity and resistance are not very well defined, nor is the antibody mechanism of immunity against the schistosomes clearly understood. A review of the literature on this subject has recently been published by Kagan (1958).

In this study two experiments were performed: Vaccination of mice with metabolic-product antigens of the life cycle stages of *Schistosoma mansoni,* and passive-transfer with serum from animals infected with *Schistosomatium douthitti.* Groups of mice were inoculated with soluble antigens prepared by incubating stages of the life cycle of *S. mansoni* in water, and then exposed to infection with *S. mansoni.* By comparing the longevity of inoculated mice with control mice, the value of the inoculum and its effect on the resistance of the host to infection were determined. In the passive transfer experiment the classical method of serum transfer was used in an attempt to protect mice against a challenge infection. In previous passive transfer studies the number of worms recovered in the immunized and control hosts was the criterion for determining the immune state (Vogel & Minning, 1953; Stirewalt and Evans, 1953; Kagan, 1958; Sadun and Lin, 1959). In the present experiment the longevity of experimental and control animals when challenged with a lethal dose of cercariae of *S. douthitti* was used to evaluate the presence or absence of resistance.

MATERIALS AND METHODS

Both cercarial and adult metabolic products were used as antigens in the vaccination experiment. Laboratory infected *Australorbis glabratus* of Puerto Rican origin was used as the snail host. The cercariae were collected from snails isolated in small beakers filled with aquarium water (which was prepared by storing tap water for several weeks in large tanks, with constant aeration). These beakers were placed under an incandescent bulb for 3 to 4 hours in the afternoon, with the temperature regulated from 30 to 37 C. Cercariae were collected in large numbers and concentrated by centrifugation at an average centrifugal force of 320 *g.* Approximately 400,000 cercariae were placed overnight in 5 to 10 ml of water in a petri dish at room temperature. The contents of the petri dish were centrifuged and the supernatant was

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frozen and stored at −20 C. This process was repeated four times, using the same thawed supernatant liquid each time so that an antigen was prepared that contained metabolic products of approximately 1.6 million cercariae. An adult metabolic-product antigen was made in a similar manner, using during each incubation period 300 male and female *S. mansoni* adult worms collected by perfusing infected hamsters. The two antigens were also combined in a 1 to 1 ratio yielding a third antigen used in the vaccination experiment.

The inocula were prepared by adding equal amounts of antigen and Freund's adjuvant (8.5 volumes of Bayol F plus 1.5 Arlocel A plus 10 mg dried BCG organisms per 100 ml of oil) and emulsifying the mixture in a Virtis homogenizer for 1 or 2 minutes.

Seventy-five mice (18 g, white females) were divided into five groups of 15 mice each. Group A was a control group which received no inoculations. Group B was a control group which received sham inoculations of water in Freund's adjuvant. Groups C, D, and E were inoculated with cercarial metabolic products, adult metabolic products, and a combination of cercarial and adult metabolic products, respectively. Intraperitoneal injections of 0.5 ml of the Freund's mixture were given to the respective groups (B, C, D, E) once a week for 5 weeks. On the sixth week, all five groups of mice were exposed for 1 hour to approximately 700 cercariae of *S. mansoni* per mouse, by immersing the tails of the mice in small glass tubes containing the cercariae in water (Olivier and Stirewalt, 1952). Deaths in each group were recorded chronologically. Student's *t* test was used to determine the significance of the results.

In the passive transfer experiment, three groups of mice were used; one received injections of normal hamster serum, one received injections of serum from hamsters infected with *S. douthitti* and one received no injections of serum. All mice were exposed to 100 cercariae of *S. douthitti* and deaths were recorded chronologically.

Normal serum was collected from hamsters 13 to 14 weeks old, pooled, and kept at −20 C. Serum was also collected from hamsters which had been exposed to 300 or more cercariae of *S. douthitti*, and which had harbored the parasites for 3 to 4 months. The serum was pooled and kept at −20 C.

Cercariae of *S. douthitti* were collected from *Lymnaea stagnalis* raised and infected in the laboratory. The snails were placed in beakers, in the dark, from 4:00 p.m. until 10:00 p.m., at which time many cercariae could be seen on the surface film of the water. The cercariae were removed from the surface film by means of a wire loop, three-eighths inch in diameter. Thirty mice were divided into three groups of 10 mice each. Mice in group A received no injections of serum, those in group B received injections of normal hamster serum, and those in group C injections of immune serum. Mice in groups B and C were injected intravenously in the tail veins with 0.25 ml of serum daily for 4 days before exposure, and with 0.5 ml of serum on the day of exposure and for 7 days after exposure. All three groups of mice were exposed to 100 cercariae of *S. douthitti* (a lethal exposure) by placing the cercariae on the shaven moistened abdomen of the mice, each of which had been immobilized by being fastened to a board with a piece of adhesive tape across the thorax and with elastic bands holding the legs outstretched (Nolan et al, 1947). A record was kept of the longevity of the mice in each group. The data were analyzed by Student's *t* test.

EXPERIMENTAL RESULTS

Vaccination experiments: Table I depicts the total number of mice which died

TABLE I. *Number of mice which died after exposure to* Schistosoma mansoni *following vaccination with cercarial and adult metabolic product antigens.*

Groups of mice	No. mice exposed	Mice dead in weeks after exposure																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
A	10								3	4	5	8	9	10						
B	9				2	3	5	5	6	6	7	9								
C	12	1	1	1	2	2	3	3	3	4	4	6	8	10	11	11	11	11	11	11
D	13				1	2	2	2	2	5	6	8	8	11	11	11	12	13		
E	11					2	2	3	3	3	5	6	8	9	9	9	11			

A, no inoculation controls.
B, water controls.
C, cercarial metabolic products.
D, adult metabolic products.
E, cercarial and adult metabolic products.

in each group (A, B, C, D, E) in weekly intervals after exposure to *S. mansoni*. In the two control groups (A and B) all the mice had died within 11 to 13 weeks after exposure, 3 to 5 weeks before all mice had died in the experimental groups (C, D, E).

In the statistical analysis of this experiment the mean week of death was computed only for those mice living longer than 7 weeks after exposure, since mice dying earlier probably died from non-relevant causes. The mouse in group C which was alive 19 weeks after exposure (table I) was assumed to have died 20 weeks after exposure in order to facilitate the computation. Mean time of death for each group of mice and the standard errors of these means are listed in table II.

TABLE II. *Statistical analysis of inoculation experiment with metabolic antigens of* Schistosoma mansoni.

	A	B	C	D	E
Group	15	15	15	15	15
No. Mice	No inoculations	Tap water	Cercarial metabolic antigen	Adult metabolic antigen	Cercarial and adult metabolic antigen
No. of mice exposed	10	9	12	13	11
No. of mice surviving 7 weeks of infection	10	4	9	11	8
Mean week of death for mice alive at 8 weeks of infection	$10.1 \pm .57$	$10.0 \pm .71$	$12.8 \pm .94$	$11.9 \pm .84$	$12.5 \pm .84$
Average mean week of death for control and experimentals	$10.1 \pm .44$			$12.4 \pm .50$	
Difference between mean			2.3 ± 0.67		
"*t*" value			3.4 (significant 1 percent level)		

Since the averages and standard errors of the two control groups (A, B) are very close and since the averages and standard errors of the three experimental groups (C, D, E) are also very close, these results were combined to give one control group and one inoculated or experimental group. For the combined control group (A, B) the overall mean week of death and standard error is 10.1 ± 0.44 weeks respectively with 13 degrees of freedom for the standard error. The overall mean week of death and standard error for the combined inoculated or experimental group (C, D, E) is 12.4 ± 0.50 weeks with 27 degrees of freedom. The means of the control and inoculated mice were compared by computing the ratio of the difference between the means to the standard error of this difference. The difference between the two means is 2.3 weeks with a standard error of 0.67. The ratio of the difference to its standard error is 3.4 which is significant at the 1 percent level. Since the mean week of death for the inoculated mice is greater than that for the control mice by an amount significant at the 1 percent level, the data suggest that immunization prolonged the life of these mice.

Passive transfer experiment: Table III indicates the comparative longevities of three groups of mice: A, which received no injections of serum; B, which received injections of normal serum; and C, which received injections of antiserum. There were no deaths in control group A until 5 weeks after exposure, after which time there was rapid death. At the termination of the experiment, 13 weeks after exposure, all the mice in both control groups (A, B) had died, whereas three mice of group C were still alive. For the statistical analysis of these data, the fifth week after exposure was taken as the start for these calculations. The three mice in group C which were alive 13 weeks after exposure were assumed to have died at

TABLE III. *Deaths of mice after exposure to* Schistosomatium douthitti *following passive transfer of immune serum.*

Groups of mice		A	B	C
No. mice exposed	No. weeks infected	10	10	10
	1		4	4
	2		2	0
	3		0	0
	4		0	0
	5		0	1
	6	4	1	2
Deaths in weeks after exposure	7	0	1	0
	8	3	0	0
	9	2	0	0
	10	0	0	0
	11	0	0	0
	12	0	0	0
	13	1	2	3*
Average time of deaths in weeks and standard error		7.9 ± 0.69	4.7 ± 1.54	6.0 ± 1.67
No. mice surviving 4 weeks of infection		10	4	6
Mean week of death for mice alive at 5 weeks of infection		7.9 ± 0.69	9.75 ± 1.89	9.83 ± 1.90

A, no transfer controls.
B, normal serum transfer controls.
C, anti-serum transfer experimentals.
* These three mice were still alive 13 weeks after exposure.

14 weeks in order to facilitate the computation. As can be seen in table III, the mean times of death of the normal serum control group (B) and the immune serum experimental group (C) are almost equal, 9.75 ± 1.89 and 9.83 ± 1.90 weeks, respectively, indicating that the injection of immune serum did not prolong the life of mice challenged with infection.

DISCUSSION

In the vaccination experiment, results indicate that immunization with cercarial and adult metabolic-product antigens conferred a certain amount of resistance to the vaccinated host as measured by increased longevity. The published work of the role of metabolic-product antigens in the immunity of *Nippostrongylus muris* (Thorson, 1951, 1953, 1954) and *Trichinella spiralis* (Campbell, 1955; Chute, 1956; Chipman, 1957) indicated a need for further study of the role of these antigens in the immunology of schistosomiasis. Sadun, Lin and Walton (1959) prepared a metabolic antigen from adult worms of *S. japonicum*. Vaccination of mice with metabolic antigen caused a statistically significant reduction in worm burden when mice were challenged with 50 cercariae (Sadun and Lin, 1959). Other workers have reported positive results in inducing resistance by vaccination with worm antigens of *S. japonicum* (Ozawa, 1930; Kawamura, 1932; Lin, Ritchie and Hunter, 1954). Watts (1949) reported vaccination with antigens of *S. mansoni* in mice, but Thompson (1954) was unable to verify her results. Kagan (1958) reported negative results for vaccination experiments with antigens of *S. douthitti*. The present experiment results indicate that protective antigenic stimulation with antigens of *S. mansoni* is possible when the proper antigens are employed.

The rationale for using the seventh week as the basis for calculating longevity in the vaccination experiment and the fifth week in the passive transfer experiment and for not calculating all deaths from the time of exposure is based on the following biological data. In the vaccination experiment mice were challenged with a lethal dose of 700 cercariae of *S. mansoni*. Mice exposed to this dose began to die

under normal circumstances the eighth week after exposure due to the passage of eggs through the walls of the intestine which results in profuse hemorrhaging (Olivier and Schneidermann, 1953). In infections with *S. douthitti* mice exposed to a lethal dose of 100 to 300 cercariae also die of hemorrhage approximately 5 weeks after infection since eggs are produced by this species approximately 30 days after exposure (Kagan et al, 1954). In both experiments reported in this manuscript animals died during the period of immunization and transfer of serum due perhaps to the effects of the Freund's adjuvant in the former instance or to the faulty injection of serum which may have caused shock by the injection of serum not brought to body temperature or the use of hamster serum in mice. To calculate a mean period of survival using the death of mice which died from causes other than the parasitic infection would bias the results. We, therefore, excluded the mice that had died prior to the seventh week and fifth week in both experiments. We believe that this is not prejudicial because approximately equal proportions of the initial number of mice died in control (5 of 19) and experimental groups (8 of 36) during this early period in the vaccination experiment.

Olivier and Schneidermann (1953) conducted a longevity experiment by infecting mice with light bisexual infections of *S. mansoni* and then challenging them later with a lethal infection of this parasite. In their table I, deaths by weeks after challenge were tabulated making an analysis of their results with our statistical method possible. In the original analysis, by averaging the survival time for immunized mice from the time of challenge, including mice that may have died from the initial exposure, their results were "not statistically significant;" however, these workers were able to show that the immunized mice did acquire an immunity as evidenced by lower worm counts in these animals as compared to controls. In regard to their longevity experiment, they stated that the two groups (immunized and controls) "differed significantly" in their survival patterns. When one excludes deaths in immunized and control groups prior to the seventh week of infection, the average length of life after challenge for the immunized mice in their experiments No. 1 to No. 4 becomes statistically significant. In their experiment No. 5 (mice initially infected with male worms) the average survival time is not significant. In their original analysis they also recognized that there was no difference in survival between immunized and controls in their experiment No. 5.

Until recently, passive transfer of immunity against schistosomes has not been demonstrated, conclusively. Kawamura (1932) reported protection in dogs and rabbits, whereas Vogel and Minning (1953), Stirewalt and Evans (1953), and Kagan (1958) reported negative results. Sadun and Lin (1959) have shown that mice injected with immune rabbit antiserum and worm extract antiserum had a statistically significant reduction in worm burden when infected with 50 cercariae of *S. japonicum;* antiserum to metabolic products did not result in a statistically significant reduction in worm burden. These data are important, because in order to place the mechanism of schistosome immunity on a firm antigen-antibody basis passive transfer of immunity must be conclusively demonstrated. Perhaps the techniques used to demonstrate passive transfer for *S. mansoni* are not adequate. Stirewalt and Evans (1953) used immune rat serum and in the present study immune hamster serum was used. Perhaps the technique of parabiosis used so successfully by Zaiman (1953) in his trichinella study would be the method to demonstrate passive transfer in *S. mansoni* infections.

SUMMARY

Two experiments on vaccination and passive transfer of immunity are reported. In the vaccination experiment mice injected with metabolic products antigens of cercariae and adult worms of *S. mansoni* showed a statistically significant longer survival period over control mice when challenged with a lethal infection of *S. mansoni*. In the passive transfer experiment mice injected with immune serum recovered from hamsters infected with *S. douthitti* and challenged with a lethal infection with this parasite did not show any difference in survival over control mice.

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INFECTIVITY OF THE PHILIPPINE STRAIN OF *SCHISTOSOMA JAPONICUM* IN *ONCOMELANIA HUPENSIS*, *O. FORMOSANA* AND *O. NOSOPHORA**

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The geographic strains of *Schistosoma japonicum* show different degrees of infectivity in various species of oncomelanian snails. Hunter et al (1952) reported that the Japanese strain of *S. japonicum* can develop well in *Oncomelania nosophora* from Japan but hardly at all in *O. formosana* from Formosa, China. DeWitt (1954) showed the following: (1) The Chinese strain of *S. japonicum* can develop in *O. hupensis* from Shanghai, China, but not in *O. formosana* from Formosa, China; *O. nosophora* from Japan, nor in *O. quadrasi* from the Philippines; (2) the Formosan strain can develop well in *O. formosana* from Formosa, China; *O. nosophora* from Japan, and to a lesser degree *O. quadrasi* from the Philippines, but not in *O. hupensis* from Shanghai, China; and (3) the Japanese strain can develop in *O. nosophora* from Japan, and *O. hupensis* from Shanghai, China, but not in *O. formosana* from Formosa, China, nor in *O. quadrasi* from the Philippines. As to the Philippine strain, although it should unquestionably infect *O. quadrasi* from the Philippines, its infectivity in the oncomelanian snails from the other endemic areas has not been studied.** In order to complete our knowledge concerning the infectivity of various geographic strains of *S. japonicum* in different *Oncomelania* species, a study of the infectivity of the Philippine strain in *O. hupensis, O. formosana,* and *O. nosophora* was initiated.

It is an unanswered question whether the *japonicum* complex is made up either by four geographic races, four subspecies of a single polytypic species, or four biological species (Hsü and Hsü, 1960). Attempts to answer this question by experimental interbreeding of different geographic strains of the parasite were undertaken in this laboratory. The miracidia of the F_1 generation, if obtained, should be tested for their infectivity in the *Oncomelania* species of both its paternal and maternal affiliations. On this account, our knowledge concerning the infectivity of the Philippine strain of *S. japonicum* in *O. hupensis, O. formosana,* and *O. nosophora* should also be completed.

MATERIALS AND METHODS

The miracidia of the Philippine strain of *S. japonicum* were obtained from eggs from liver and intestine of mice infected with the cercariae shed from *O. quadrasi* collected in Leyte, the Philippines. Laboratory-reared *O. hupensis* originating in Canton, China, and *O. formosana* originating in Formosa, China, were used. These snails were about 1 year old. As to *O. nosophora,* infection was made with field snails collected from Kofu, Japan. They had been proved

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** Wagner and Chi (1959) misquote DeWitt's (1954) work that the Philippine strain of *S. japonicum* develops only in the Philippine snail, *O. quadrasi.*

negative from previous infection by 3 months of repeatedly negative cercariae-shedding tests in the laboratory.

During the infection, each snail was kept in a small dish 14 mm in diameter. Only those snails which showed definite activity were used. About 10 newly hatched miracidia in two drops of fresh river water were given to the snail in the dish. After 15 minutes, three additional drops of water were added. The snail was pushed back into the water whenever it crawled out. After an hour, the exposed snails were transferred back to their original aquaria which were installed in a room with constant temperature of 27 C. Five weeks after the exposure, cercariae-shedding tests were made weekly for 14 weeks. The snails were considered positive if any *japonicum* cercariae were shed during this period, and negative if no cercariae were found. In order to be certain that infection did not exist, all the negative snails were crushed at the end of the 14-week period and examined for the presence of immature cercariae.

RESULTS

In the present series of experiments, 43 *O. hupensis,* 880 *O. formosana,* and 200 *O. nosophora* were exposed to infection with miracidia of the Philippine strain of *S. japonicum.* The exposure of *O. hupensis* and *O. nosophora* to infection was made on the same day, whereas the exposure of *O. formosana* was made in three batches (125 snails in the first batch, 135 in the second, and 620 in the third) on 3 different days. The results of the experiments are given in table I. While *O. formosana* was

TABLE I. *Results following exposure of* Oncomelania hupensis, O. formosana, *and* O. nosophora *to the Philippine strain of* S. japonicum.

	O. hupensis	*O. formosana*	*O. nosophora*
Snails exposed	43	880	200
Snails survived	40	730	125
Snails shedding cercariae	8	0	12
Additional positive snails after crushing	0	0	0
Percent of positive snails	20	0	9.6

refractory to infection with Philippine miracidia, 20 percent of *O. hupensis* and 9.6 percent of *O. nosophora* became infected.

DISCUSSION

The results of the present study show that the Philippine strain of *S. japonicum,* like the other geographic strains, manifests different degrees of infectivity with *Oncomelania* species from different geographic areas. It is appropriate now, for the sake of comparison, to summarize in one table (table II) the results of the studies

TABLE II. *Percent of infection of* O. hupensis, O. formosana, O. nosophora, *and* O. quadrasi *exposed to four geographic strains of* S. japonicum *as reported by various investigators.*

Strain of *S. japonicum*	*O. hupensis*	*O. formosana*	*O. nosophora*	*O. quadrasi*
Chinese	34 (D) 23–80 (V)	0 (D)	0 (D)	0 (D)
Formosan	0 (D)	35 (D)	21 (D)	0.4 (D)
Japanese	13 (D)	0 (D) 0.8 (HRO)	21 (D) 44.4 (HRO)	0 (D)
Philippine	20 (HH)	0 (HH)	9.6 (HH)	44–75 (P)

(D) : DeWitt, 1954.
(HH) : Present authors.
(HRO) : Hunter, Ritchie, and Otori, 1952.
(P) : Pesigan, Hairston, Jauregui, Garcia, Santoes, Santos, and Besa, 1958.
(V) : Vogel, 1958.

on the infectivity of different geographic strains of *S. japonicum* in different *Oncomelania* species as obtained by various authors. It is of interest to see that each geographic strain of *S. japonicum* infects best the *Oncomelania* species from its own endemic area, and to a lesser degree or not at all infects the snail species from the

endemic areas of the other strains. The Chinese strain is the most typical in this respect. It can infect only *O. hupensis* from the Chinese mainland. The other three strains are evidently less specific. There is, however, no strain which is so versatile as to infect all four species of *Oncomelania.* The data in table II indicate two facts: (1) The four geographic strains of *S. japonicum* differ biologically in their infectivity with the four *Oncomelania* species, and (2) the four geographic species of *Oncomelania* also differ biologically in their susceptibility to the four strains of *S. japonicum.*

The origin of the four geographic strains of *S. japonicum* has been discussed in our previous paper (Hsü and Hsü, 1960). We are of the opinion that the origin of the four *Oncomelania* species followed the same pattern. Thus, both the ancestral *S. japonicum* and *Oncomelania* species originated in the main part of the Old World tropics and from there radiated into China, Japan, Formosa, Celebes, and the Philippines. We do not know when *Oncomelania* first became the intermediate host of *S. japonicum* and mammals and man its final host. As soon as *S. japonicum* became an obligate parasite, its geographic distribution had to be determined by the geographic distribution of the hosts. The final hosts of this parasite, man, domestic mammals, and rodents, are present ubiquitously in Asia while the *Oncomelania* snail has a special geographic distribution. Consequently *S. japonicum* became endemic only in those areas where the *Oncomelania* snail could establish itself.

As the ecological conditions and general adaptations were different in different isolated areas, the characteristics of various strains of *S. japonicum* and various species of *Oncomelania* in different islands, that is, Japan, Formosa, Celebes, and the Philippines, should have developed differently. The insular strains of *S. japonicum* have become distinct in physiological characteristics, although not morphologically; and the insular species of *Oncomelania* became distinct not only physiologically but also morphologically, although genetically interspecies breedings are still possible (Komiya and Kojima, 1958; Wagner and Chi, 1959). In this way, four distinct geographic strains of *S. japonicum,* the Chinese, Formosan, Japanese, and Philippine, and four geographic species of *Oncomelania, O. hupensis, O. formosana, O. nosophora,* and *O. quadrasi,* have been formed.

In the course of millions of years of selection and adaptation, the vector-parasite compatibility has reached its optimum condition between the local species of *Oncomelania* and local strain of *S. japonicum.* The data given in table II for the relative infectivity-susceptibility of the four geographic strains of *S. japonicum* and four geographic species of *Oncomelania* illustrate the results of this evolutionary process.

SUMMARY

1. *Oncomelania hupensis, O. formosana,* and *O. nosophora* were exposed to the Philippine strain of *Schistosoma japonicum.* While *O. formosana* was refractory to infection, 20 percent of *O. hupensis* and 9.6 percent of *O. nosophora* became infected.

2. The results of the studies on the infectivity of different geographic strains of *S. japonicum* in different *Oncomelania* species obtained by various authors are summarized in one table. The relative infectivity-susceptibility of the four geographic strains of the parasite and four geographic species of the intermediate hosts illustrates the results of millions of years of evolutional adaptation.

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RESEARCH NOTE

ANTHELMINTIC TRIALS WITH HYGROMYCIN IN PIGS.

Critical and controlled tests were undertaken with cull pigs in an attempt to assess the efficacy of hygromycin, an antibiotic exhibiting anthelmintic properties in swine (Goldsby and Todd, 1957, No. Am. Vet. **38:** 140). The treatment regimen was the same in all trials: the principals were maintained on full feed for 35 days on a ration containing 12 million units of hygromycin, or 5 pounds Hygromix (Lilly) per ton of feed. Since other workers have reported that the material also affects the growth rate of swine (Kelley et al, 1958, Vet. Med. **53:** 120), records were kept on weight gains and feed consumption. The results may not be highly significant because the pigs available for most of the trials were constitutionally inferior.

Hygromycin exhibited anthelmintic action, but had no apparent effect on weight gains in trials with two groups of seven newly weaned pigs. At necropsy, only 10 ascarids (1 mature, 9 immature) and 1 whipworm were recovered from the principals as compared with 85 ascarids (5 mature, 80 immature) and 18 whipworms from the controls. Initially, the average weights were 27.3 pounds for the principals and 27.1 pounds for the controls; after 35 days, they averaged 58.0 and 60.1 pounds, respectively. The total feed consumption was the same for both groups. Therefore, hygromycin exhibited no favorable influence on weight gains in these trials.

Substantial anthelmintic action was indicated also in a similar trial with two groups of five pigs. At necropsy, only 17 ascarids and 3 whipworms were recovered from the medicated group whereas 395 ascarids and 313 whipworms were harbored by the controls. Lungworms were found in four of the five principals and in three of the five controls. Feed conversion data showed that the principals and controls required, respectively, 331 and 336 pounds of feed per 100 pounds gain in live weight.

Limited anthelmintic data obtained in critical tests with hygromycin were somewhat less impressive. In one trial, the feces from two groups of four pigs were examined daily for worms during the entire experimental period. In 35 days, the treated group apparently eliminated 70 percent (9) of 13 ascarids and 11 percent (29) of 263 whipworms, whereas 1 percent (1) of 98 ascarids and 10 percent (11) of 114 whipworms were recovered from the feces of the untreated controls. The data on ascarids suggest the possibility that additional worms may have been eliminated in the medicated group and eaten by the pigs before they could be collected. It seems unlikely, however, that this would account for the poor efficacy against whipworms, reputedly one of the three species most amenable to the anthelmintic effects of the material.

One control pig died during the test period, presumably from general debilitation. Moreover, none of the pigs, principals or controls, showed reasonable weight gains, possibly because of the poor condition of the animals and not from parasitic infections. Feed utilization data, therefore, were not significant.

In a single critical test, the regimen removed 90 percent (48) of 54 ascarids, 70 percent (201) of 288 whipworms, and all of 7 nodular worms from a pig that weighed 76 pounds when the trial was initiated. During the test period, this animal gained 69 pounds, or one pound per 3.4 pounds of feed.

The results indicate that more data are needed on the anthelmintic action of hygromycin in swine as well as on its effect on growth rate and feed conversion.—M. L. COLGLAZIER AND F. D. ENZIE, *Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.*

PRELIMINARY STUDIES ON THE LIFE HISTORY OF *CARDIOCEPHALUS BRANDESI* SZIDAT, 1928 (TREMATODA: STRIGEIDAE)

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During several summers prior to 1956 specimens of the trematode *Cardiocephalus brandesi* Szidat, 1928 (Strigeidae), often were recovered from young laboratory hatched and reared birds which had been fed small fish; hosts included skimmers (*Rhynchops nigra nigra*), common terns (*Sterna hirundo hirundo*) and least terns (*S. albifrons*). In 1955, for example, a total of 47 *C. brandesi* were recovered from five laboratory-raised common terns which had been fed these small fish indiscriminately. Subsequently, carefully controlled experiments were run with laboratory-hatched birds in order to identify the larval forms of *C. brandesi.*

Routine examination of silversides, *Menidia menidia* (Linn.), and mullets, particularly *Mugil cephalus* Linn., revealed large numbers of developing larvae showing morphological affinities to *C. brandesi.* The larvae were found primarily in the brains of these fish, but not infrequently the eyes were sites of infection. The silversides carried a much heavier infection than the mullets; eight *Menidia* ranging in size from 4.0 to 7.5 cm had from 12 to 108 developing worms in the ventricles of their brains. (It is of interest to note that the largest fish, with 108 *Cardiocephalus,* also had two encysted gasterostome larvae within the brain case, but not the brain.) All ages of larvae were found in these natural infections; feeding experiments revealed, however, that encysted forms only were infective and there were relatively few encysted worms in the infected fish (fig. 1). Some unencysted worms grow to a comparatively large size; failure to encyst may be due to overcrowding or other unknown physiological factors.

The following is a summary of the 1956 experiments. Five laboratory-hatched skimmers were fed only *Cyprinodon variegatus;* three of these birds were completely negative at autopsy 5 and 7 days after feeding began. Two, however, while negative for *Cardiocephalus,* had one and three, respectively, young *Phagicola sp.* (Heterophyidae). The first host died 75 hours after feeding began and the second died after 48 hours. Whole young mullet, *M. cephalus,* were fed to five other skimmers; two of these birds were completely negative, but these hosts died within 26 hours after their first feeding. Eleven *Cardiocephalus* were recovered from the third skimmer after 100 hours. Approximately 50 worms were taken from the fourth when autopsied at 13 days. The recovered worms were of all ages and one shed eggs when put into water. Several worms were used in oxygen-consumption studies; however, 31 were preserved immediately after recovery. The fifth skimmer had 10 young *Cardiocephalus,* the maximum age possible being 18 days, the

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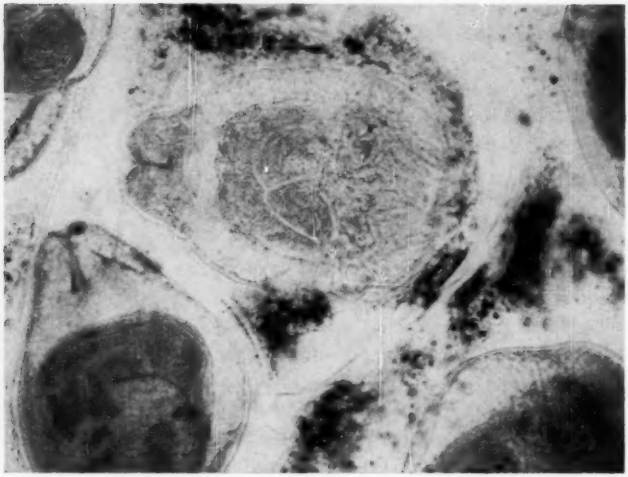

FIGURE 1. Encysted metacercariae of *C. brandesi* from brain of *Menidia menidia*. Approx. 100 ×.

minimum being 48 hours.

During the summer of 1959, realizing the *Menidia* were much more heavily infected than mullet, a second series of experiments was attempted with known numbers of worms fed to two skimmers and one common tern. The largest of the metacercariae were taken from the *Menidia* brains and given to the laboratory-hatched birds maintained on a controlled diet of dog food which contained no fish. Three such experiments, in which unencysted larvae were fed, yielded no results; this indicated that the metacercariae were non-infective before cyst formation. The time factor prevented us from dissecting brains and feeding known numbers of cysts; therefore, whole brains were fed. Using 4-day-old laboratory raised chicks as hosts, five worms were recovered at 72 hours from one, 11 after 7 days from one, and none from two others on the 6th and 15th days. The bird autopsied on the sixth day had been force-fed with the brains in a physiological saline solution; the other birds were given the brains as they were lifted from the fish. The negative results obtained with the 15-day autopsy may be due to the length of the experiment as chicks are not normal hosts. All of the chicks had been fed 20 whole brains except this last one which had taken 30 brains.

The above data present positive identification of larval *Cardiocephalus brandesi* in fish intermediate hosts. Ground work has been accomplished for detailed developmental studies.

Cercaria nassa Martin, 1945, occurs frequently in *Nassarius obsoleta* in the Beaufort, N. C., area. Martin (1945) suggested the probability of this cercaria developing into a member of the Strigeidae, but he reported unsuccessful attempts to infect *Fundulus heteroclitus, F. majalis,* and *Paralichthyes dentatus.*

Penetration experiments involving *F. heteroclitus* and *F. majalis* in our laboratory corroborated Martin's results. However, successful penetration into small mullets was accomplished and larvae were recovered from these hosts as early as

10 hours after exposure. More than 50 such larvae were removed and preserved as follows: 0 to 10 hrs, several; 0 to 24 hrs, 2; 45 to 69.5 hrs., 3; 89 to 113 hrs, 7; 163 to 187 hrs, 8; 9 to 10 days, 9; 18 to 19 days, 15. The longest exposure was 2 days with the fish in aquaria with actively emerging cercariae. When the eyes were the sites of infection, the larvae could be seen without dissection when observed by means of an ordinary dissecting microscope.

Unsuccessful attempts at penetration were observed when the cercariae attacked the general body surface. Several observations were made of successful penetration through the roof of the mouth, however. Entrance through the gills also was suspected, but actual observations of penetration were confined to the mouth pathway. Encysted forms of *Cardiocephalus* have been recovered from the brains only, and worms indistinguishable in size and morphology from *C. nassa* have been recovered and photographed.

Menidia were not used in these penetration experiments because of their failure to survive in the laboratory. *Lucania parva* (Baird and Girard), the rainwater fish, attracted *C. nassa,* and penetration was observed; however, no viable cercariae were recovered even when the larvae reached the eyes.

Further work is necessary to complete and substantiate the picture of this part of the life history of *Cardiocephalus brandesi.* Evidence herein presented, however, indicates the strong possibility of *Cercaria nassa* being its cercarial stage.

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ON *OGMOCOTYLE INDICA* (BHALERAO, 1942) RUIZ, 1946 (TREMATODA), THE NOTOCOTYLID MONOSTOME OF INDIAN OVINES, AND ITS PATHOGENICITY

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Bhalerao (1942) first reported *Ogmocotyle indica* as a common parasite in the small intestine of ovines and named the species *Cymbiforma indica*. Ruiz (1946) reduced the genus *Cymbiforma* Yamaguti, 1933, to synonomy with *Ogmocotyle* Skrjabin and Schultz, 1933, and recognized three species: the genotype, *O. pygari* Skrjabin, 1933; *O. sikae* (Yamaguti, 1933); and *O. indica* (Bhalerao, 1942). Price (1954) described a fourth species, *O. ailuri* (erroneously named *Ogmogaster ailuri*). Skrjabin (1953) reduced *O. sikae* to synonymy with *O. pygari*, but Yamaguti (1958) recognized all four species.

Ogmocotyle indica was considered a harmless parasite until Katiyar (1956) reported outbreaks of a serious disease of sheep on some U.P. farms and ascribed it to heavy infections with that trematode under the name cymbiformiasis. The disease was characterized by muco-purulent enteritis with superficial necrosis, marked cellular reaction with eosinophilic infiltration, and also focal invasion of the mucosa by leucocytes and eggs of the parasites; the worms were free, adhered to the intestinal lining or were embedded in its wall. Mixed infections of *Dicrocoelium dendriticum*, trichostrongyles, and *O. indica* were present in some animals with oedematous submandibular swellings, but because the last species predominated, symptoms were attributed largely to it.

MATERIALS AND OBSERVATIONS

During November 1959 one of us (Bhatia) examined hill sheep from U.P. adjacent to Tibet and slaughtered at Nainital. Massive infections of *Ogmocotyle indica* were found in the small intestine. The previous month, that trematode was observed also in a hill goat examined at Pithoragarh (Almora district). In practically all cases the infection was a mixed one, with large numbers of *Trichostrongylus colubriformis* and *Strongyloides papillosus*. Most of the sheep were adults and in good condition.

Pieces of duodenum and jejunum with the attached parasites were prepared as serial sections to study the histopathology of the infection and the biology of the parasite. The worms were mostly free in the mucus and contents of the jejunum; whereas in the duodenum, they fastened themselves to the general mucosa (fig. 1a) or extended into the crypts to become attached (fig. 1b) and thus account for some of the damage attributable to attachment.

The body of the trematode consists of an elongated, translucent anterior portion with well developed unicellular glands near the esophagus and a larger posterior region which is boat-shaped and, in living specimens, is greyish lightly tinged with pink and with a prominent white speck due to the uterus filled with eggs. The genital pore is at the junction of the two parts and the sides of the posterior region

FIGURE 1. Photomicrographs of sectioned duodenum showing *Ogmocotyle indica* (a) in slightly oblique section, with a plug of musoca in the groove between the two body regions; and (b) in longitudinal section within a duodenal crypt and showing a similar plug (× ca 53).

which are ventrally incurved are more strongly so at that level to grip the mucosa of the intestine. As the worm moves from place to place to feed and fasten itself, patches of mucosa may become detached to account for all of the injury that could be ascribed to it from the study of serial sections. There was no evidence that the parasite penetrates the intestinal wall far enough to become embedded or that its eggs infiltrate the mucosa. However, nematodes were present and extensive damage such as desquamation, erosion, congestion, and cellular infiltration of the mucosa and submucosa was caused by the tunneling activities of the parasitic females of *S. papillosus* and penetration by both sexes of *T. colubriformis* which may reach the lamina propria.

Our observations indicate that *O. indica* alone causes no more than mild abnormalities of the duodenum resulting from the parasite's attachment and feeding activities whereby patches of the mucosa may be destroyed. The more severe effects attributed to that species by Katiyar (1956) are due to mixed infections with nematodes, influenced perhaps by such predisposing factors as climate, stress, and malnutrition. Whether mixed infections enhance the damage done by a particular species remains to be determined by controlled experimental infections.

SUMMARY

The pathogenecity which Katiyar (1956) attributed to *Ogmocotyle indica* in sheep and goats is due instead to mixed infections with nematodes. The trematode itself causes no more than moderate damage resulting from attachment and feeding by the worm whereby patches of the intestinal mucosa are destroyed.

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RESEARCH NOTE

A RAPID METHOD FOR CLEARING AND MOUNTING THE GENITALIA OF FEMALE IXODID TICKS.

Adler and Feldman-Muhsam (1948, Parasitology **39**: 95–101) reported that the genital aperture of female ticks provides a valuable taxonomic feature for identification of ticks of the genus *Hyalomma*. Subsequently, this characteristic has been utilized by several workers for diagnosing species of other ixodid genera.

The method of preparing the genitalia for study advocated by Adler and Feldman-Muhsam was as follows: Ticks were placed in lactophenol for 24 hours and then transferred to 2 percent potash for 3 to 5 days. They were then passed through water, alcohols, and clove oil, and mounted in Canada balsam. Later Feldman-Muhsam (1956, Bull. Res. Coun. of Israel **5B**: 300–306) used a slightly different technique: The genitalia were removed from the female, cleared in 2 percent KOH for 24 hours, washed in water, dehydrated in alcohol, and mounted in balsam.

These procedures have several disadvantages: They are time consuming for routine identification; the prolonged exposure to KOH leaves the genitalia soft and difficult to manage; and adequate control cannot be exercised over the clearing process when it is carried out over such an extended period of time. Accurate control over the clearing process is desirable in order to allow for variation in sclerotization of different specimens.

The need for a faster and more efficient procedure was noted while exploring the possibility of using the genitalia to determine females of the genus *Rhipicephalus* found in a large collection of ticks from the Belgian Congo.

The following method gave the desired results: The genitalia are removed with a corneal knife and placed in a 1-cc beaker containing a small amount of 100 percent lactic acid. The lactic acid is then heated to just below the boiling point for from 1 to 3 minutes, depending on the sclerotization of the specimen, and the amount of clearing desired. Following this the specimen is washed in water in order to avoid crystallization of any excess lactic acid. At this time the muscles attached to the vaginal tube are carefully removed with the aid of fine forceps. The specimen is oriented in a drop of Hoyer's medium and a cover-slip is added. The preparation is then ready for examination with a compound microscope.

This procedure has several advantages: The time required for removing and preparing genitalia for examination is only 15 minutes; lactic acid is more gentle in its action than KOH; and clearing can be accurately controlled since the specimen can be examined conveniently at any time during the process.

In order to insure that the results of the shorter method are comparable to those of Feldman-Muhsam, 20 specimens were selected from a reared series of *Rhipicephalus simus* and 10 were cleared and mounted by each method. A comparison of these specimens failed to disclose any significant differences. Therefore, no advantage can be seen in using the longer method.

We should like to thank Dr. Gertrud Theiler of the Veterinary Laboratory, Onderstepoort, Union of South Africa, for loan of the laboratory-reared specimens used in this work. This project was supported by the Research and Development Division, Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-981 with the University of Maryland.—CARLETON M. CLIFFORD AND DONALD T. LEWERS, *Department of Zoology, University of Maryland, College Park, Maryland.*

STUDIES ON THE MORPHOLOGY AND LIFE-HISTORY OF *NOTOCOTYLUS MINUTUS* N. SP., A DIGENETIC TREMATODE FROM DUCKS

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The U. S. Fish and Wildlife Service is perturbed because of the reduction in numbers of the soft-shelled clam, *Mya arenaria,* along the coast of New England (Glude, 1955). The writer is studying the parasites of the clam and of its predators (Stunkard, 1951, 1953, 1957, Stunkard and Uzmann, 1958) in an attempt to discover the causes of the decline and possible biological measures for control of the principal predators, the green and horseshoe crabs, *Carcinides maenas* and *Limulus polyphemus. Mya arenaria* harbors the asexual generations of digenetic trematodes, including the sporocysts and cercariae of *Cercaria myae* Uzmann, 1952. These larvae were shown by Stunkard and Uzmann (1958) to be developmental stages of a species of *Gymnophallus,* whose sexual generation occurs in shore-birds. The asexual stages invade the digestive and sexual organs of the mollusk, limiting or terminating its reproductive activity.

The clams also serve as intermediate hosts of other trematodes and 3 species of metacercariae occur commonly in the gills and palps. The life-cycles and bionomics of these worms are now under investigation. Since the asexual generations of these metacercariae must occur in mollusks which live in the immediate vicinity of the infected clam, an attempt is being made to find and identify them. Furthermore, since the definitive hosts of these metacercariae are animals that feed on *M. arenaria,* examination of shore-birds has been started. To obtain information on the sexually mature stages, metacercariae have been fed to laboratory-raised birds: chicks, eider ducks, herring gulls and common terns, as well as to mice and hamsters. The clams and other mollusks, the eider ducklings, and a sampling of shore-birds were provided by Walter R. Welch, Chief of Clam Investigations, Boothbay Harbor, Maine, under whose authority the study is conducted. Grateful appreciation is here expressed to him and members of his staff.

One of the most heavily parasitized snails is *Hydrobia minuta.* Examination of this snail disclosed infection by a notocotylid species, member of a group in which the cercariae encyst on objects in the water. The number of cercariae liberated by such a small snail is not large, the incidence of infection is low, about 0.1 to 0.2 percent, and since the larvae encyst soon after emergence, the finding of swimming cercariae is unusual. These cercariae encysted on the shells and opercula of *H. minuta* and on empty shells of *Gemma gemma* were fed to laboratory-reared gulls (*Larus argentatus*) and to eider ducks (*Somateria mollissima*). Adult worms recovered from the eider proved to be morphologically identical with others taken 30 years ago from the entrails of an unidentified duck and described by Stunkard

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and Dunihue (1931) as *Notocotylus gibbus* (Mehlis in Creplin, 1846). These specimens were assigned by Harwood (1939) to *Notocotylus imbricatus* (Looss, 1893), a species based on *Cercaria imbricata* Looss. It is now manifest that the worms do not belong to either *N. gibbus* or *N. imbricata;* they are described as a new species, *Notocotylus minutus.*

The measurements recorded are in millimeters.

Notocotylus minutus n. sp.
Syn. *Notocotylus gibbus* Stunkard and Dunihue, 1931.
Notocotylus imbricatus ex parte, Harwood, 1939.

The Adult (Fig. 4)

Sexually mature specimens measure 1.0 to 1.5 in length and 0.40 to 0.60 in width. The type specimen, shown in figure 4, from the cecum of a young eider duck, is somewhat flattened and measures 1.15 long and 0.50 wide. The body is flattened; the sides almost parallel; convex dorsally and concave ventrally. There are scattered bits of pigment in the anterior region as far back as the beginning of the metraterm. In the description by Stunkard and Dunihue (1931) the statement was made that there are 3 rows of ventral pits, 12 to 14 in each row. Harwood (1939), who studied those specimens, reported 15 in the median and 16 in the lateral rows. The worms had been taken from a duck, long dead, and were in poor condition. It is often difficult to determine the number of glandular pits on the ventral side of the body, especially at the posterior end which may be curved to form a cup-like lip that obscures the structure. In the specimen shown in the figure, there are clearly 16 pits in each of the 3 rows. Whether or not the number is variable or the apparent differences are merely the result of failure to see one or more of the structures is yet uncertain.

The oral sucker measures up to 0.12 in diameter; there is no pharynx; the esophagus is short and the digestive ceca are irregularly narrow in diameter. In the anterior half of the body they are median to the lateral pits while in the posterior portion they extend above the glands to the region of the gonads, where they turn mediad and pass between the testes and the ovary, terminating near the posterior end of the body.

The testes are situated on opposite sides at the posterior end of the body. They are deeply lobed, 0.20 to 0.25 long and 0.12 to 0.14 in greatest breadth. Ducts from the testes unite in front of the ootype to form a common duct which passes forward on the dorsal side of the body. It becomes coiled as it approaches the middle of the body and expands to form a seminal vesicle. Immediately in front of the uterine loops it enters the cirrus sac and may expand again inside the sac. The cirrus sac measures from 0.45 to 0.56 in length; the genital pore is below the esophagus.

The ovary is situated between the digestive ceca at the level of the testes. It is deeply lobed from 0.12 to 0.15 in diameter. The ootype complex and shell gland are located anterior to the ovary. The uterus extends in many transverse loops between the digestive ceca to the level of the posterior end of the cirrus sac, then forward beside the cirrus sac to the metraterm, which is about one-half the length of the cirrus sac. The eggs measure from 0.02 to 0.024 by 0.01 to 0.012; they bear long polar filaments at both poles.

The Redia (Fig. 3)

The rediae are sausage-shaped to cylindrical, often attenuated at one or both ends. Those with developing cercariae measure from 0.38 to 1.00 long and 0.14 to 0.30 in greatest width; others are smaller. The pharynx measures 0.028 to 0.034 in diameter; the intestine is short and saccate; it contains greenish to yellowish material, obviously digested snail tissue. The number of rediae in a snail is small, usually about a dozen, of different sizes. The small ones are motile, the larger ones lose motility as they become filled with progeny; however, the number of cercariae is relatively small, often only 1 or 2 cercariae and many younger stages in a redia.

The Cercaria (Figs. 1, 2)

The cercariae measure from 0.16 to 0.29 in length but can extend under pressure of a cover-glass to a length of 0.34. Ordinarily the width is about one-half the length, although the width varies inversely with the length as the larva elongates and contracts. The oral sucker is 0.023 to 0.026 in diameter although it becomes apparently larger under compression. The esophagus is about the length of the oral sucker and the ceca end blindly near the posterior end of the body. Brownish to blackish pigment begins to form in young cercariae, especially around the anterior end, and increases in amount as the larvae mature. There are 2 eye-spots, 0.014 to 0.017 in diameter, provided with lenses, and situated 1 on either side above the commissure of the

nervous system. Concentration of pigment may at times suggest a smaller third one between the two. Strands of pigment extend backward, typically 3 on each side as shown in figure 1. There are gland cells which open near the oral sucker and many cystogenous cells, scattered throughout the body. The tail is small, typically thin and tapering, 0.03 to 0.035 wide at the base and 0.20 to 0.40 long. The tip of the tail may form a small muscular papilla. There are small locomotor pockets, situated ventrally on either side of the tail, but often completely hidden by the glandular cells that fill the area. The pattern of flame-cells and tubules was obscured by development of glands and only the major components of the excretory system were observed. The collecting ducts unite at the level of the eye-spots to form a finger-like projection which may extend to the level of the oral sucker. The ducts thus form a loop which becomes filled with concretions as the cercaria matures. The excretory vesicle is small and the common duct, formed by fusion of primary ducts, extends far into the tail where separation of the primary ducts restores 2 tubules which lead to the primary excretory pores (fig. 2). The form of the second vesicle, produced by fusion and enlargement of the collecting ducts, places this cercaria in the Yenchingensis Group of Miriam Rothschild (1938).

The Metacercaria (*Fig 5*)

The cercariae encyst soon after emergence and were taken from the opercula and shells of *H. minuta* and from empty, washed shells of *G. gemma*. The cysts are circular, 0.12 to 0.16 in diameter. They are flattened on the attached side and convex on the opposite side. The wall is 0.014 to 0.020 thick. The larvae do not grow in the cysts and measurements of the oral sucker were 0.024 to 0.029. The worms retain the pigment but the eye-spots are not distinct in specimens that have been encysted for some weeks.

DISCUSSION

Since the report by Stunkard and Dunihue (1931), knowledge of species in the genus *Notocotylus* has been substantially increased but the situation remains complicated and confused. By feeding young, parasite-free ducklings, L. and U. Szidat (1933) reported that *Cercaria ephemera* Nitzsch, 1807 from *Planorbis corneus* developed into adults which, in violation of the rules of nomenclature, they designated as a new species, *Notocotylus thienemanni*. They also reported that cercariae from *Lymnaea palustris* became adults which were identified as *Notocotylus attenuatus* (Rudolphi, 1809). The authors noted that in poorly preserved and distorted specimens it is difficult if not impossible to make accurate counts of the ventral glandular packets, but that in living specimens it is clear that the number and arrangement of these structures are constant and that they can serve as useful characters for specific identification. *Notocotylus gibbus* was redescribed. In this species the glandular packets are arranged with 11 in each lateral row and 4 larger ones in a median row. With the more complete description of *N. gibbus* it is apparent that the specimens described by Stunkard and Dunihue (1931) differ from that species in hosts and geographic distribution, in size of worms, size and shape of gonads, and in number and arrangement of glandular packets. Ursula Szidat (1935) fed larvae from *Bithynia tentaculata*, which were identified as *Cercaria imbricata* Looss, 1893, to chicks and ducks and recovered adults strikingly similar to *N. attenuatus*, but designated erroneously as a new species, *N. imbricatus*. The species was characterized by the small size of the cercariae and the identity of the intermediate host; Szidat remarked on the difficulty of distinguishing between these closely related trematodes on purely morphological grounds. Ursula Szidat (1936) insisted on the marked specificity of larval stages and the snail host, and listed 11 species of *Notocotylus*. She proposed a new genus *Barkeria* to contain *Notocotylus quinqueserialis*, an action that was anticipated by the erection of *Quinqueserialis* Skvortsov, 1935, and a new genus *Kossackia* to contain *N. gibbus*. Lal (1935) had named *N. gibbus* as type of a new genus, *Hindia*.

Harwood (1939) gave a comprehensive review of the subfamily Notocotylinae with keys to the genera and species. He discussed the morphology and taxonomy of species in the genus *Notocotylus,* rejected the proposed genera *Hindia* and *Kossackia,* and added 4 new species from North American hosts. Under the heading *Notocotylus imbricatus* (Looss, 1893), Szidat, 1935, Harwood included *Monostoma* sp. Stiles and Hassall, 1894; *Cercaria imbricata* Looss, 1893; *Notocotylus seineti* Fuhrmann, 1919; *Notocotylus urbanensis* Harrah, 1922, in part; *Notocotylus gibbus* Stunkard and Dunihue, 1931 (not Mehlis in Creplin, 1846); *Notocotylus intestinalis* Tubangui, 1932; *Notocotylus orientalis* Ku, 1937; *Hindia lucknowensis* Lal, 1935; and *Notocotylus anatis* Ku, 1937. A comparative table listed the characteristics of *N. imbricatus* from the various sources. Examination of that table discloses a heterogeneous collection of similar but not identical worms, from varied hosts and different parts of the world. The specimens described by Stunkard and Dunihue are distinct as the smallest members of the collection. *Notocotylus imbricatus* is based on *Cercaria imbricata* Looss as type. But the identity of *C. imbricata* is not clear. Miriam Rothschild (1940) pointed out that *C. imbricata* Looss, 1893, from *Paludina impura* (= *Bithynia tentaculata*) taken near Leipzig, Germany was named provisionally in a footnote without adequate description and, therefore, is a nomen nudum. Looss (1896) described as *C. imbricata* a cercaria from *Melania tuberculata* Egypt; the description of the species is concerned with the Egyptian and not the German cercaria. Miss Rothschild observed, p. 215, "It seems very probable that Looss' original surmise was correct, and that the cercaria he discovered in *Paludina impura* was the larval form of a *Notocotylus* identical with, or very closely related to, *N. attenuatus,* whereas the species from *Melania tuberculata* was the larval form of *Catatropis verrucosa.*" This opinion is supported by the report of U. Szidat (1935) who fed specimens from *Bithynia tentaculata* to chicks and ducks and obtained worms that were exceptionally like *N. attenuatus.*

Herber (1955) studied the life-history of *Notocotylus urbanensis* (Cort, 1914). He noted errors in the accounts of Barker (1915) and Harrah (1922); stated that the species described by Barker in the text as *Catatropis fimbriata* and figured as *Catatropis filamentis* and the species described by Harrah as *Paramonostomum echinatum* were based on poorly prepared specimens of *Notocotylus urbanensis* and that both names should be suppressed as synonyms. According to Herber, the specimens described by Harrah (1922) as *Notocotylus urbanensis* are not conspecific with *Cercaria urbanensis* Cort, 1914, but comprise a heterogeneous collection of immature specimens and the only one identified were referred to *Quinqueserialis.*

The determination of species in the genus *Notocotylus* is difficult. The worms are very similar and specific differences in morphology are small. Host specificity of the adult stages is not definite, because the same metacercariae can mature in a number of different host species. Apparently the snail hosts are more specific, and U. Szidat (1935) predicated precise specificity in *N. ephemera, N. attenuatus,* and *N. imbricata.* But there is so much confusion in the identification of larvae that final conclusions are impossible. No less than 4 different cercariae have been described as larvae of *N. attenuatus* by Joyeux (1922), Mathias (1930), L. and U. Szidat (1933), and Yamaguti (1938).

Although the identity of *N. imbricata* is yet problematical, it is clear that the worms described by Stunkard and Dunihue (1931) and those now at hand, whose

larval stages are in the marine snail, *Hydrobia minuta*, are not identical with either of the species reported by Looss, that they are not members of *N. imbricata*, and represent a previously unnamed species, which is now designated *Notocotylus minutus*. The type specimen is deposited in the Helminthological Collection of the U. S. National Museum under the number 39,409.

SUMMARY

The asexual generations of a species of *Notocotylus* occur in the marine snail, *Hydrobia minuta*, in the region of Boothbay Harbor, Maine. Encysted metacercariae developed to maturity in a young, laboratory-reared eider duck. These worms are morphologically identical with others described by Stunkard and Dunihue (1931) as *Notocotylus gibbus* and assigned to *Notocotylus imbricatus* by Harwood (1939). New information discloses that the specimens cannot be assigned to either *N. gibbus* or *N. imbricatus*, and they are described as a new species, *Notocotylus minutus*. Redial, cercarial, metacercarial, and adult stages are figured.

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EXPLANATION OF PLATE

All drawings are of fixed and stained specimens, with certain details added from the study of living worms.

FIGURE 1. Cercaria, showing extent of pigmentation and position of the digestive organs; body of specimen, 0.2 mm long.

FIGURE 2. Cercaria, showing major features of the excretory system, the Anlagen of the gonads as a mass of germinal cells just anterior to the excretory vesicle, also the location and extent of the cystogenous glands; body of specimen, 0.23 mm long.

FIGURE 3. Redia; 0.8 mm long.

FIGURE 4. Adult specimen, from a laboratory raised eider duck, ventral view, showing principal nerve trunks, ventral glandular pits, digestive and reproductive organs. The body is concave ventrally and the posterior end is curved upward, partially obscuring structures in this region; specimen 1.15 mm long.

FIGURE 5. Metacercaria, from the operculum of *Hydrobia minuta*, showing oral sucker, pigmented area, and concretions in the excretory tubules; diameter of cyst, 0.15 mm.

RESEARCH NOTE

COMPARATIVE TRIALS WITH SOME PRESENT-DAY ANTHELMINTICS FOR SWINE.

Five groups of five cull pigs each were used in limited trials to determine the comparative efficacy of four common swine anthelmintics. The products employed, given in accordance with accepted procedures, were cadmium oxide (0.015 percent of ration for 3 days), sodium fluoride (1.0 percent of dry, ground feed for 1 day), piperazine citrate (50 mg piperazine base per pound of body weight in about three-fourths of 1-day's feed consumption), and hygromycin (12,000,000 units per ton of feed for 5 weeks); the fifth group was used as untreated controls. All pigs were comparably infested, as far as could be determined by pre-treatment egg counts, and all groups were randomized. The feces were not screened to recover parasites removed by the various treatments; rather, the results were based upon necropsy worm counts at the end of a 35-day test period. Table I gives the average worm counts of the groups.

TABLE I. *Average worm counts at necropsy.*

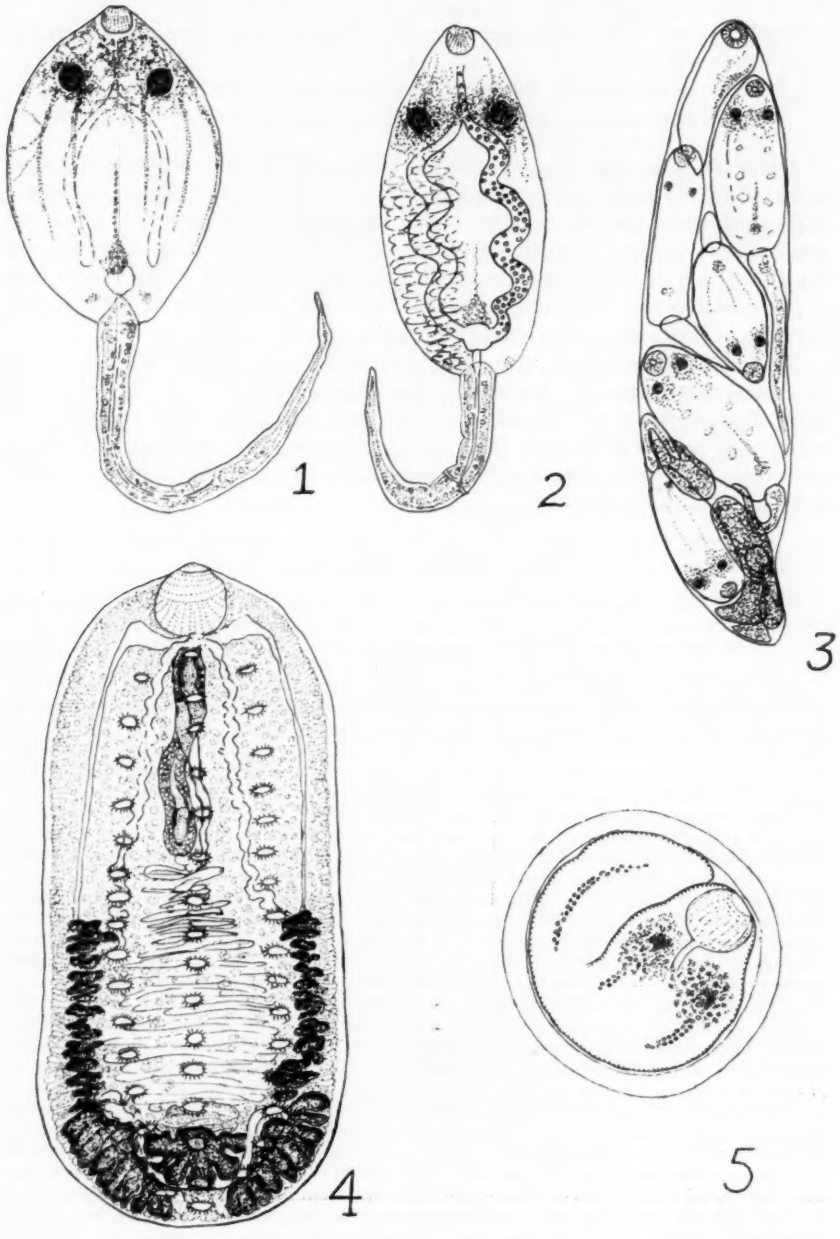
Group	Treatment	Ascarids	Parasites	
			Nodular worms	Whipworms
1	Cadmium oxide	84	33	163
2	Sodium fluoride*	2	2	267
3	Piperazine citrate**	6	1	131
4	Hygromycin ***	2	0	20
5	None (untreated controls)	90	112	156

* One pig died after 19 days; ** one pig died after 22 days; *** one pig died after 21 days (4 ascarids and 35 whipworms recovered at necropsy are not included in calculations).

The data suggest that efficient control of ascarids and nodular worms may have been achieved with sodium fluoride, piperazine citrate, and hygromycin, but there was little evidence that cadmium oxide was effective against these parasites. Hygromycin was the only product that appeared to be effective against whipworms. Inasmuch as the principal and control groups involved only five animals each, these interpretations are tentative, although the differences approach significance at the 5 percent level ("t" test).

Several of the principals showed little change in weight at the end of the test period; and one pig died from each of the three groups receiving sodium fluoride, piperazine citrate, and hygromycin, presumably from general debilitation. Because of the constitutionally inferior animals involved, data on feed consumption, weight gains, and feed conversion efficiency were of little or no significance.—MERLE L. COLGLAZIER AND FRANK D. ENZIE, *Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Dept. of Agriculture, Beltsville, Maryland.*

PLATE I



EURYTREMA PROCYONIS DENTON, 1942 (TREMATODA: DICROCOELIIDAE), FROM THE DOMESTIC CAT

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Eurytrema procyonis was described by Denton (1942) from racoons in Texas. Stunkard (1947) found a species in a red fox from New York State which he described and named *Eurytrema vulpis*. Later, on comparing his specimens with some received from Denton, Stunkard concluded that they were of the same species and that *E. vulpis* is a synonym of *E. procyonis* (Stunkard and Goss, 1950). Since then *E. procyonis* has been found in a racoon in Connecticut (Penner, et al, 1954) and in racoons and gray foxes in Maryland (Herman, et al, 1957).

On July 31, 1959, a stray cat from New Jersey was examined in this Laboratory for intestinal helminths and dicrocoeliid-type eggs were found in the feces. At autopsy 67 small flukes were found in the bile ducts and gall bladder and over 300 were tightly packed in the pancreatic ducts. (Another stray cat from New Jersey taken on February 29, 1960, was found at autopsy to have 28 *Eurytrema procyonis* in the distal portion of the pancreatic duct.)

Description of Eurytrema procyonis *from the Cat*
(Measurements in millimeters)

Body foliaceous, margins entire, about half as thick as wide, maximum width in hindbody. Living specimens 2.57 to 3.29 long by 1.06 to 1.38 wide; fixed and stained, 2.00 to 2.30 by 0.86 to 0.97. All measurements that follow are from mounted material. Cuticle thin and smooth, except for a few sensory papillae on anterior part of body. Oral sucker subterminal, subspherical, 0.158 to 0.181 long by 0.177 to 0.196 wide, with dorsal lip-like projection above. Acetabulum subspherical, weakly muscular, within a fibrous capsule, 0.251 to 0.299 long by 0.244 to 0.303 wide, in anterior half of second quarter of body. Sucker ratio 1:1.44 to 1:1.61, average 1:1.52. Pharynx, 0.102 to 0.114 long, 0.107 to 0.124 wide; esophagus generally S-shaped in somewhat vertical plane, bifurcation about one-seventh body length from anterior end; ceca fluctuating in width, 0.060 to 0.090 in diameter, terminating about one-seventh body length from posterior end. Excretory pore terminal, vesicle saccate, reaching about to level of posteriormost vitelline follicles. Main (or primary) collecting tubules extend from antero-lateral margins of vesicle to mid or posterior level of testes, where each receives an anterior and posterior secondary tubule. Testes, two, symmetrical, spherical to subspherical, 0.134 to 0.191 long by 0.138 to 0.186 wide, immediately posterior or touching but not overlapping acetabulum, contiguous or slightly separated. From dorso-medial margins of testes, the vasa efferentia extend anteriorly, then medially to unite as they enter cirrus sac. The cirrus sac, 0.258 to 0.330 long, 0.117 to 0.155 wide, ovoid, extending from genital pore to overlap acetabulum dorsally, ending at about midlevel of that sucker. Seminal vesicle, ejaculatory duct and cirrus within cirrus sac. Genital atrium midventral, at or slightly posterior to intestinal bifurcation. Ovary spherical, 0.108 to 0.157 in diameter, posttesticular, submedian to directly posterior to either testis, which it may touch or be separated from by its diameter. Seminal receptacle spherical, 0.062 to 0.105 in diameter, dorsal to ovary, eccentric to it in any direction in the horizontal plane. Mehlis' gland large, diffuse, median or opposite to ovary, overlapping its level or a short distance posterior to that level. Laurer's canal extends dorsally and anteriorly to open near posterior level of testes. Vitellaria extracecal, sometimes partly ventral to ceca, consisting of numerous small, rounded follicles, irregularly arranged, extend from posterior level of testes to anterior level of excretory vesicle. Vitelline ducts pass mediad, ventral to ceca, to unite and form the common vitelline duct in the median line. Uterus, with many coils and loops, extending first from the ootype to the posterior end of the body and then anteriorly above and between the testes and dorsal to the cirrus sac to open at the genital atrium. Eggs oval, operculate, yellowish brown, rarely flattened on one side, 0.052 to 0.059 long by 0.033 to 0.038 wide.

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DISCUSSION

The major differences between *E. procyonis* from the cat and from the racoon and red fox concern the reproductive system. Flukes from the cat have smaller, spherical, testes (about 0.160 in diameter) that do not overlap the acetabulum, whereas in material from the racoon and fox, the testes are oval, larger (about 0.270 to 0.290 by 0.180 to 0.190) and overlap the acetabulum. Also, the ovary is small (0.080 to 0.090) and round or oval, whereas it is spherical and large (about 0.130) in flukes from the cat. They also have a somewhat larger seminal receptacle diameter (about 0.086), in contrast to one of about 0.065 in the racoon and about 0.032 in the fox. The eggs in the flukes and in the cat's feces were somewhat larger than the measurements given for the species from other hosts. However, some of the differences given above may be correlated with physiological differences in the racoon, fox, and cat.

Travassos (1944) and Yamaguti (1958) placed *Eurytrema procyonis* in the genus *Concinnum,* which, according to Yamaguti, has an acetabulum usually larger than the oral sucker, postequatorial vitellaria and a pre-bifurcal genital pore. In *Eurytrema,* the acetabulum is nearer the size of the oral sucker, the vitellaria are largely postequatorial and the genital pore is postbifurcal.

A comparison of these three features in 30 flukes from the cat shows that they agree with the genus *Concinnum* only in having the acetabulum average about 50 percent greater in diameter than the oral sucker. In all but one specimen, the vitellaria begin in the preequatorial region, even though most of the follicles are postequatorial; the distance of the anteriormost follicles from the anterior end was from 42.5 to 50.5 percent of the body length. In none was the genital pore prebifurcal; it was ventral to the bifurcation in approximately half of the flukes and posterior to the bifurcation of the remainder. The species thus shows 2 features of *Eurytrema* and 1 of *Concinnum,* thus adding support to Stunkard's observation (1947) that *Concinnum* and a number of other dicrocoeliid genera are not distinguished readily from *Eurytrema.*

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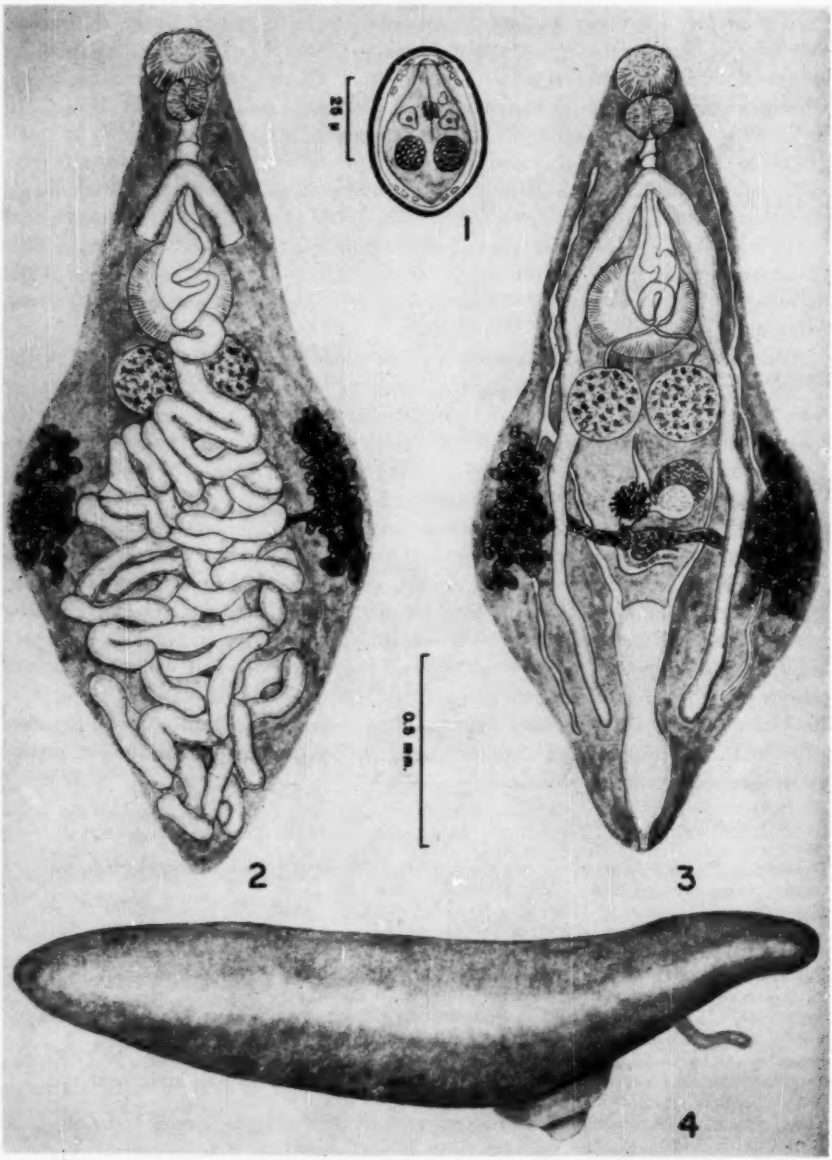
PLATE I



EXPLANATION OF PLATE I

FIGURE 1. Egg of *Eurytrema procyonis*.

FIGURE 2. Whole mount of fluke, dorsal view, to show extent of uterus. Eggs are omitted from uterus.

FIGURE 3. Whole mount of fluke, dorsal view, to show reproductive systems, except for most of uterus, excretory system and digestive system.

FIGURE 4. Lateral view of relaxed and unstained fluke to show thickness of body, appearance of relaxed acetabulum, and extrusion of cirrus.

PHYSA SAYII, A NEW INTERMEDIATE HOST FOR THE TURTLE LUNG FLUKE, *HERONIMUS CHELYDRAE* (TREMATODA: HERONIMIDAE)

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Iowa State University and Iowa Lakeside Laboratory

The turtle lung fluke, *Heronimus chelydrae* MacCallum, is frequently encountered as a parasite of painted turtles (*Chrysemys picta belli*) and snapping turtles (*Chelydra serpentina*) in the numerous lakes of northwest Iowa. Recent studies on details of sporocyst development of this fluke have been presented by Ulmer and Sommer (1957), and an account of the life cycle was presented by Crandall (1959, 1960.)

As reported in 1957 by Ulmer and Sommer, *Physa gyrina* serves as a suitable experimental and natural host for this species. A series of 25 experiments conducted in 1956, involving 114 snails of seven different species, resulted in the recovery of sporocysts only from *Physa gyrina*, the most abundant physid of the Okoboji region. Crandall's studies (1960) involved *Physa integra* Haldeman as an intermediate host. The abundance of *Physa sayii* Tappan in Lake Okoboji suggested the desirability of further experimental work, despite the negative findings of 1957 when attempts to infect this species with *Heronimus* miracidia met with no success. At that time, two experiments were conducted, involving single and multiple exposures of miracidia to 10 *P. sayii*.

During the summer of 1959 at Lake Okoboji, a series of 50 laboratory-reared physids (*P. sayii* and *P. gyrina*) were employed in laboratory-induced infections (table I). In every instance, snails were exposed to large numbers of miracidia.

TABLE I. *Development of* Heronimus chelydrae *sporocysts* (*June to July, 1959*).

Snail host	No. snails exposed to miracidia	No. snails examined	No. snails infected	Length of snails	No. sporocysts recovered	Age of sporocysts
Physa gyrina	36	1	1	40 mm	1	10 days
		1	1	16 mm	1	18 days
		5	1	15 mm	1	24 days
		6	2	12 mm	1	31 days
				15 mm	4	31 days
Physa sayii	14	6	1	6 mm	11	24 days
			1	10 mm	1	36 days

Nineteen snails survived during the course of the experiment, seven of which became infected.

Two of the six *P. sayii* exposed to miracidia developed typical *Heronimus* sporocysts possessing actively pulsatile lateral branches. Sporocysts were recovered 24 and 36 days, respectively, after exposure of snails to miracidia. In one snail, 24 days post-exposure, 11 fully formed sporocysts were found; in the second, a single sporocyst was recovered (fig. 1). Cercarial development in both instances was well advanced. Five of 13 surviving *P. gyrina* were positive for *Heronimus* sporocysts, varying in age from 10 to 31 days.

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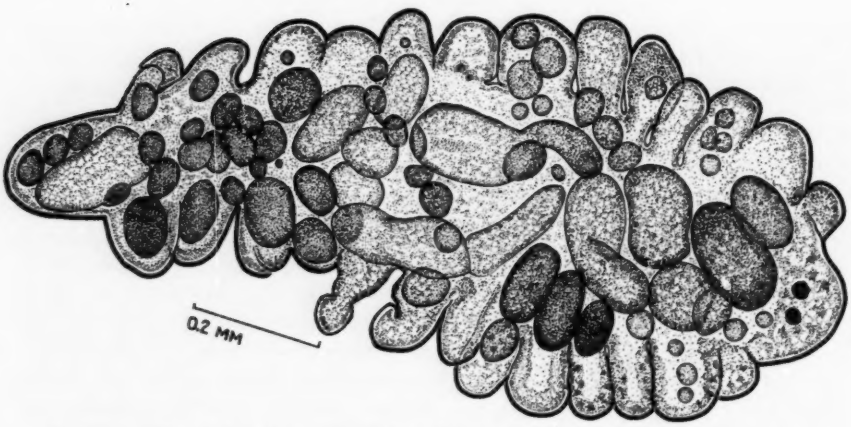

FIGURE 1. Twenty-four-day sporocyst from *Physa sayii*.

Size of the molluscan host, both in the case of *P. sayii* and *P. gyrina*, apparently has little effect on the susceptibility of the gastropods, for development occurred in larger snails (up to 40 mm) as well as in smaller ones (6 mm).

At least three species of physids, namely *P. gyrina*, *P. integra*, and *P. sayii* are thus known to be capable of harboring the sporocyst stages of this fluke.

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TELORCHIS BONNERENSIS N. SP. (TREMATODA: DIGENEA) FROM THE INTESTINE OF LARVAL *AMBYSTOMA MACRODACTYLUM* BAIRD, FROM NORTHERN IDAHO

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During a survey of helminth parasites of amphibians and reptiles in Idaho, 294 digenetic trematodes were recovered from the intestinal tracts of several larval long-toed salamanders, *Ambystoma macrodactylum,* and one garter snake, *Thamnophis sirtalis.* The worms were assigned to the genus *Telorchis* Luhe, 1889; since they differ from other members of the genus, the name *T. bonnerensis* n. sp. is proposed for them.

RESULTS

Because the worms become ovigerous when only one-third their maximum size, measurements in the following diagnostic description are given first as averages from 10 fully grown worms, and then in parentheses as ranges from 25 worms of all sizes containing eggs. All measurements are in millimeters.

Telorchis bonnerensis n. sp.

Description. Slender, elongate distomes; length 4.83 (1.60 to 5.25), width 0.502 (0.196 to 0.530). Cuticle thin, covered with minute spines posteriorly to the level of the acetabulum. Oral sucker subterminal, round, without lappets, 0.149 (0.090 to 0.153) in diameter; prepharynx short, pharynx 0.087 (0.043 to 0.089) wide and 0.061 (0.035 to 0.071) long; esophagus 0.084 (0.057 to 0.89) in length; intestinal ceca extending to the posterior end of the body. Acetabulum round, always smaller than oral sucker, median, or slightly to one side, 0.137 (0.065 to 0.142) in diameter. Ovary just within anterior one-third of body length, oval, 0.187 (0.072 to 0.203) long and 0.150 (0.043 to 0.163) wide. Seminal receptacle present; descending and ascending limbs of uterus confined to intercecal space and ending in a muscular metraterm approximately one-half the length of cirrus sac. Vitellaria in ungrouped follicles, mostly extra-cecal, beginning near anterior margin of acetabulum and stopping short of anterior testis by approximately twice its length. Testes near posterior end of body, nearly equal in size, tandem, oval; anterior testis 0.289 (0.100 to 0.313) long and 0.194 (0.072 to 0.232) wide; posterior testis 0.319 (0.082 to 0.360) long and 0.206 (0.072 to 0.220) wide. Cirrus sac 0.65 (0.30 to 0.69) long, extending to the anterior edge of the ovary; cirrus protrusible. Eggs oval, 0.042 (0.039 to 0.044) by 0.019 (0.018 to 0.021).

Hosts: *Ambystoma macrodactylum* Baird, 1849 (larvae only), *Thamnophis sirtalis* L.
Site: small intestine.
Type locality: Clarksfork, Bonner Co., Idaho.
Deposited specimens: Type and paratypes #39415, U.S.N.M. Helm. Coll.

DISCUSSION

The status of the genus *Telorchis* is disputed. Lühe (1900) recognized two genera, *Telorchis* and *Cercorchis.* Goldberger (1911) considered *Cercorchis* as a subgenus. Stunkard (1915) felt that *Cercorchis* was not a valid genus. Perkins (1928), however, again raised *Cercorchis* to generic status. Wharton (1940) and, more recently, Yamaguti (1958) placed all members of the genus *Cercorchis* in *Telorchis.*

Telorchis bonnerensis belongs to the subgenus *Cercorchis.* It most closely re-

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sembles *T. corti* Stunkard, 1915, but differs in the anterior extent of the vitellaria, distance of the ovary from the cirrus sac, and in egg size. The vitellaria are distributed as in *T. cryptobranchi* McMullen and Roudabush, 1936, but the acetabulum of *T. bonnerensis* is always smaller than the oral sucker, its eggs are longer, and the vitelline follicles are not grouped. It resembles *T. necturi* Perkins, 1928, and *T. stunkardi* Chandler, 1923, in having an amphibian host, but differs in having an acetabulum which is always smaller than the oral sucker. *T. stunkardi* also has a larger oral sucker, acetabulum, and testes, as well as vitellaria organized into distinct lobes. *T. necturi* also differs from *T. bonnerensis* in width, extent of vitellaria, and in having the testes compressed.

The anterior extent of the vitellaria, length of cirrus sac, position of ovary, length of intestinal ceca, extreme posterior placement of the testes, and the size of its eggs distinguish *T. bonnerensis* from all other species in the genus.

Telorchis bonnerensis grows considerably after egg production begins. Young salamander larvae tend to harbor worms as short as 1.6 mm with the uteri filled with eggs. The largest specimens were always obtained from older larval salamanders. Adult *Ambystoma macrodactylum,* from ponds where heavily infected larvae were collected, did not harbor the parasite. The change in diet of the host with metamorphosis possibly produces changes in the microenvironment of the small intestine, resulting in loss of the parasite.

T. bonnerensis was not present in four other species of salamanders (adult *Taricha granulosa* (Skilton, 1849), adult and larval *Ambystoma tigrinum* (Green, 1825), adult and larval *Dicamptodon ensatus* (Eschscholtz, 1833), and adult *Plethodon vandykei idahoensis* Slater and Slip, 1940) collected in the same area.

SUMMARY

Telorchis bonnerensis n. sp. is described from the intestine of larval *Ambystoma macrodactylum* and one garter snake, *Thamnophis sirtalis.* The species is differentiated from others of the genus; the unusual size variation of the worm as well as its specificity are noted.

ACKNOWLEDGMENTS

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EXPLANATION OF PLATE I

Figures drawn with the aid of a camera lucida.

FIGURE 1. *Telorchis bonnerensis* n. sp., ventral view of whole mount.

FIGURE 2. *Telorchis bonnerensis* n. sp., detail of acetabular region.

Abbreviations: C, cirrus; CS, cirrus sac; GP, genital pore; MET, metraterm; OV, ovary.

RESEARCH NOTE

PROTOSCHELOBATES SP., AN ORIBATID MITE FROM INDIA, AS A POTENTIAL VECTOR OF THE SHEEP TAPE WORMS *MONIEZIA BENEDENI* AND *M. EXPANSA*.

While working on the life-cycle of a certain anoplocephaline tapeworm during 1950 to 1953, the author had the opportunity of collecting mites from the lawn in the vicinity of Zoology Department, University of Aligarh, India. Two types of mites were encountered in the collection. They have been identified by Dr. E. W. Baker of the Entomology Research Division, U. S. Department of Agriculture, as belonging to the genera, *Protoschelobates* and *Trichoribates*. These mites were exposed to the eggs of *Moniezia benedeni* and *M. expansa* in separate culture tubes under laboratory conditions of 69 F and 50 to 55 percent relative humidity. Each culture tube, containing 200 to 250 mites, was closed with a cork through which was inserted a capillary tube for the passage of air. Eggs separated from the gravid proglottids were mixed with a small quantity of sterilized sheep feces before introducing them into the culture tubes. Bits of grass leaves and roots were also added to serve as resting places for the mites. Occasionally a few drops of water were placed on pieces of filter paper kept inside the tubes to provide moisture.

Examination of the mites for larvae was started on the fifth day after exposure to the eggs and was continued for 6 weeks. The mites were crushed on a slide by gently tapping the coverslip with a dissecting needle and examined under a microscope. It was found that *Protoschelobates* became infected with larvae of both species of *Moniezia* and that about 90 percent of the mites were infected. The infection was so heavy that some of the mites contained 20 to 30 cysticercoids. No attempt was made to carry the infection to the definitive hosts.

The genus *Trichoribates*, on the other hand, was found to be unsuitable for the larval development of these cestodes.

Forty species of mites of the superfamily Oribatei have been incriminated as vectors of 13 species of cyclophyllidean tapeworms (Allred, 1954, Utah. Acad. Sci., Arts and Letters, Proc. **31**: 44–51; Baker and Wharton, 1952, An Introduction to Acarology, Macmillan Co.; Kates and Runkel, 1948, Proc. Helm. Soc. Wash. **15**: 18–33). However, the genus *Protoschelobates* seems not to have been reported as an intermediate host for the species, *M. benedeni*. Although *P. seghettii* is known to be one of the vectors of *M. expansa* in U. S. A. (Runkel and Kates, 1947, Proc. Helm. Soc. Wash. **14**: 64–67), this appears to be the first report from India. Examination of mites for studying the natural infection with these cestode larvae did not yield positive results. Nevertheless, the nature of infection under the laboratory conditions suggests that *Protoschelobates* mites can serve as vectors of the cysticercoid larvae of the two species of *Moniezia*.

The author wishes to extend his thanks to University of Aligarh for a research fellowship, to Dr. M. A. Basir Khan for the help he has received from him, and to Dr. Baker for identifying the mites.—A. M. NADAKAL, *Department of Biology, Immaculate Heart College, Los Angeles.*

PLATE I

FIG. 1

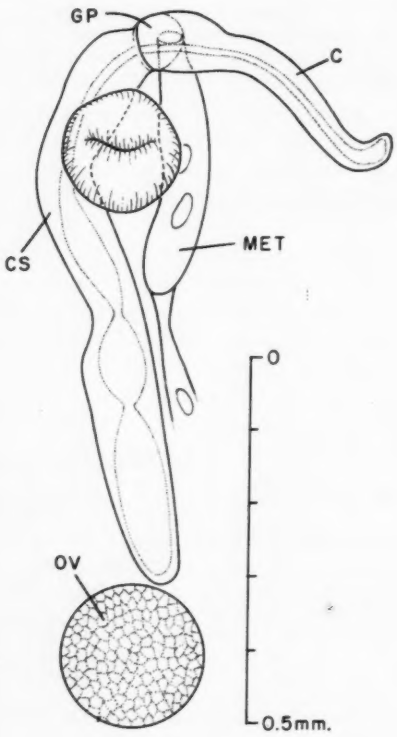

FIG. 2

ON THE LIFE HISTORY OF *MESOSTEPHANUS KENTUCKIENSIS* (CABLE, 1935) N. COMB. (TREMATODA: CYATHOCOTYLIDAE)*

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A survey of snails from streams in central Ohio revealed that *Cercaria kentuckiensis* was common in one of the prosobranch snails, *Goniobasis livescens*. *C. kentuckiensis* was first reported by Cable (1935), and later described by him (1938) from cercariae obtained from *Goniobasis semicarinata* which he collected from Scaffold Cane Creek in Madison County, Kentucky. Cable reported that the cercariae encysted in several species of cyprinid fishes. *C. kentuckiensis* was again reported by Cable (1937) and by Anderson (1944) in *Goniobasis* sp. and *Goniobasis depygis*, respectively, from McCormicks Creek, Indiana. It was again reported by Vernberg (1952) from *Goniobasis livescens* collected from Clifty Creek in southern Indiana. She found that, although the cercariae penetrate several species of fish, they encyst only in a single species of darter, the name of which was not given.

C. kentuckiensis was observed by me to penetrate and encyst in laboratory raised guppies, *Lebistes reticulatus*. The metacercariae were fed later to a starved 1-day-old chick. Seven days after the feeding, eggs of a trematode appeared in the feces of the chick and 3 days later 13 adult worms of the genus *Mesostephanus* were recovered from the small intestine of the chick. Other chicks fed this species of metacercaria from either experimental or natural hosts yielded flukes identical to the 13 recovered previously.

This paper is the result, therefore, of an investigation of the life history of a cyathocotylid trematode, *Mesostephanus kentuckiensis* (Cable 1935) n. comb. No life histories have been reported for species of the genus *Mesostephanus*, although Lutz (1935) expressed the opinion that his *Cercaria utriculata* might be the larva of 1 of 2 species of *Mesostephanus* found in sea birds in Brazil.

OBSERVATIONS AND RESULTS

The Life Cycle

The First Intermediate Host

It was determined that the snail *Goniobasis livescens* is probably the only natural first intermediate host in central Ohio for the cercaria of *Mesostephanus kentuckiensis*. An examination of 1880 individuals of this species of snail collected over a period of 1 year in central Ohio revealed that 62 (3.18 percent) were infected with this cercaria. One of 34 specimens of *G. livescens* collected from Clifty Creek, Bartholomew County, Indiana, was infected.

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The Second Intermediate Host

In the known life cycles of cyathocotylids, the second intermediate hosts have been found to be either fish, amphibia or snails. In addition to common guppies, *Lebistes reticulatus,* some tadpoles of *Rana clamitans* and *R. catesbeiana* and adult specimens of *R. pipiens* were exposed to the cercaria of *M. kentuckiensis.* Cercariae readily penetrated and encysted as metacercariae in all of these animals.

Examination of fish and tadpoles from areas in which a high incidence of infected snails occurred revealed that many species of fish and amphibians were naturally infected with the metacercariae. The cysts of this fluke are easily distinguished from cysts of other strigeids and from gasterostome larvae, which were also common in the fish examined, by their size, subspherical shape, and lack of pigment cells surrounding the cyst.

There follow, respectively, the species of fishes taken from the Olentangy River in Columbus, Ohio, and the number of each sample found to be infected with the metacercariae of *M. kentuckiensis:* Family Ictaluridae—*Ictalurus natalis* (yellow bullhead), 1 of 1; Family Cyprinidae—*Campostoma anomalum* (stoneroller minnow), 3 of 3; *Cyprinus carpio* (carp), 1 of 1; *Notemigonus crysoleucos* (golden shiner), 0 of 1; *Notropis cornutus* (common shiner), 2 of 5; *Notropis spilopterus* (spot-fin shiner), 0 of 2; *Pimephales notatus* (bluntnose minnow), 1 of 1; *Semotilus atromaculatus* (creek chub), 1 of 1; Family Clupeidae—*Dorosoma cepedianum* (eastern gizzardshad), 0 of 4; Family Centrarchidae—*Ambloplites rupestris* (northern rockbass), 14 of 25; *Lepomis macrochirus* (northern bluegill sunfish), 4 of 12; *Micropterus dolomieu* (smallmouth blackbass) 4 of 6; *Pomoxis annularis* (white crappie), 1 of 3; Family Percidae—*Etheostoma caeruleum* (rainbow darter), 0 of 2; *Etheostoma zonale* (banded darter), 0 of 2; *Percina caprodes* (logperch darter), 0 of 1.

Tadpoles of *Rana clamitans* and *R. catesbeiana* and a single specimen of *Micropterus dolomieu,* from Big Walnut Creek, located on U. S. Route 33 south of Columbus, Ohio, were examined and found to be infected with the metacercariae of this trematode. A tadpole of *R. clamitans* from the Olentangy River was heavily infected.

The Definitive Host

The cercaria of *M. kentuckiensis* most closely resembles the cercariae of *Szidatia joyeuxi* and *Prohemistomum chandleri.* Since the adult of *S. joyeuxi* occurs naturally in water snakes in Tunisia and since Vernberg (1952) recovered immature specimens of *P. chandleri* 3 days after feeding metacercariae to a *Natrix sipedon,* water snakes collected along the banks of the Olentangy River were examined for cyathocotylids. Three *Natrix sipedon* and two *N. septemvittata* were found to be uninfected. A specimen of *Natrix sipedon* was fed two small naturally infected *Ambloplites rupestris,* but was found to be negative for flukes upon postmortem examination 6 days later.

Anderson and Cable (1950) found that chicks which had never received food could be infected with the cyathocotylid, *Linstowiella szidati,* but they were unable to infect chicks which had been previously fed.

After determining that chicks would become infected with *Mesostephanus kentuckiensis* when fed the metacercariae, a number of feedings were undertaken in which naturally and experimentally infected fish and tadpoles were fed to chicks.

The results of these feedings to the chicks are tabulated in table I.

Of a total of 30 chicks fed metacercariae, 7 were found infected with immature and 7 others with the adult stage. By examining the chicks for flukes at varying periods of time after feeding them metacercariae, it was determined that this fluke matures and commences egg laying in 6 to 8 days. Fecal examination for

TABLE I. *Results of feeding experiments using 1-day-old chicks as definitive hosts.*

Host of metacercaria (N, Natural; E, Experimental)	Age of metacercaria (days)	Interval after feeding (days)	Worms recovered	
			Immature	Adult
Ambloplites rupestris N	?	2	1	0
" "	?	4	6	0
" "	?	4	0	0
" "	?	5	0	0
" "	?	10	0	0
" "	?	10	0	0
Micropterus dolomieu N	?	9	0	0
" "	?	10	0	1
" "	?	10	0	0
Lebistes reticulatus E	12	6	0	0
" "	12	16	0	0
" "	12	16	0	0
" "	22	6	0	0
" "	22	6	0	0
" "	26	8½	0	0
" "	28	4	1	0
" "	29	4	3	0
" "	29	5	5	0
" "	29	5	3	0
" "	29	5	0	0
" "	29	10	0	13
" "	36	9	0	0
" "	36	10	0	0
" "	36	24	0	[a]
" "	39	12	0	3
" "	39	15	0	[b]
Tadpole of *R. clamitans* E	46	3	2	0
" " " "	46	9	0	0
" " " "	46	10	0	2
" " " "	46	17	0	1

[a] Eggs appeared in the feces 7 days until 17 days after feeding the metacercariae.
[b] Eggs appeared in the feces 7 days until 14 days after feeding the metacercariae.

eggs confirmed this observation, eggs being found in the feces of three of the chicks on the seventh day after feeding them metacercariae.

Data obtained from three experimentally infected chicks indicated that the life span of the adult of *M. kentuckiensis* is between 14 and 17 days in this host. The feces of the three infected chicks were examined daily for trematode eggs. In the feces of one, eggs were not found 14 days after feeding the metacercaria. In the feces of a second chick, no eggs were found after 17 days. The intestine of a third chick was examined 17 days after feeding, at which time an abrupt decline in the number of eggs in the feces was observed. A single specimen was recovered in the lower third of the small intestine instead of the usual first third. The above relationships suggest that the fluke recovered in the lower third of the small intestine was a moribund individual in the process of leaving the host.

Feedings of experimentally infected guppies conducted to determine the age at which the metacercariae in the guppy became infective indicate that metacercariae become infective between 26 and 28 days after establishing in the guppy. However, in view of the fact that only a single feeding was made with metacercaria that were 26 days old, the age of infectivity may be between 22 and 28 days.

After determining the approximate age that metacercariae become infective, additional feedings of metacercariae to reptiles known to be free from strigeoid

infection and laboratory reared mammals were undertaken. A *Natrix septemvittata* fed a guppy with metacercariae 40 days old and a *N. sipedon* fed a tadpole with metacercariae 85 days old were uninfected when examined 16 days after feeding. Two laboratory mice fed guppies containing metacercariae that were 46 days old were uninfected when examined 12 days after feeding. Examination of two raccoons 8 days after feeding each an infected tadpole of *R. clamitans* revealed no cyathocotylid worms.

Comparison and Discussion

The natural first intermediate hosts of the cercaria of *M. kentuckiensis* are snails of the genus *Goniobasis,* family Pleuroceridae. No other cyathocotylid cercariae have been described from snails of the genus *Goniobasis.* However, the cercaria of *Prohemistomum chandleri* is from the snail *Pleurocera acuta,* also of the family Pleuroceridae.

The cercaria of *M. kentuckiensis* penetrates, encysts, and develops in many species of fish and amphibians. Only one other cyathocotylid, *Szidatia joyeuxi,* is known to encyst in both fish and amphibians.

The metacercaria of *M. kentuckiensis* becomes infective for chicks after 22 to 28 days encystment in the experimental second intermediate host, *Lebistes reticulatus.* This infective age agrees with the period of time required for maturation of the metacercariae of other species of cyathocotylids for which the interval of development is known.

Metacercariae of *M. kentuckiensis,* encysted for 90 days, were found to be similar morphologically to metacercariae that were only 30 days old. Komiya (1939) found that the metacercariae of *Paracoenogonimus ovatus* were still infective after 1 year.

In experimentally fed chicks, eggs of *M. kentuckiensis* first appeared after 7 days. The time between the feeding of the metacercariae and the production of eggs by the adult worms seems to vary considerably among the different species of cyathocotylids. For *Cyathocotyle orientalis* in ducks, Yamaguti (1940) found eggs after 80 hours. At the other extreme, Joyeux and Baer (1934, 141) recovered immature worms of *Szidatia joyeuxi,* none containing eggs, 9 and 11 days following the feeding of the metacercariae to the snake *Tropidonotus viperinus.* The latter instance is set apart from the others since the definitive host is poikilothermic.

While the natural definitive host of *M. kentuckiensis* has not been disclosed, the fact that chicks harbor the adult worms, suggests a probable avian definitive host. Failure of the snakes and raccoons to become infected seems to preclude the possibility of their being natural definitive hosts.

Descriptions of Stages in the Life Cycle

(Measurements are in microns unless otherwise stated)

The Egg (fig. 1)

Eggs recovered from freshly deposited feces of chicks were filled with vitelline cells, and each contained an unsegmented ovum, usually located near the opercular end. Eggs were incubated in a variety of ways, none of which resulted in the hatching of miracidia. No real development occurred inside eggs observed daily for 3 weeks.

Eggs oval, operculate, light yellow. Twenty eggs from feces of infected chicks 95 to 114 (103) by 63 to 75 (67), operculum 21 by 4. Shell often thickened on anopercular end.

The Sporocysts (fig. 2)

Over half of the 62 snails, from which cercariae of *M. kentuckiensis* were emerging, were crushed and examined for mother sporocysts. In none of these were mother sporocysts found.

Elongate, hollow daughter sporocysts of *M. kentuckiensis* were recovered from the digestive gland and also the tissues of the upper branchial cavity of infected snails. Twenty-one were dissected from one snail. The body cavity of the second generation sporocyst is filled with germinal masses and cercariae in various stages of development. In sporocysts obtained from snails crushed within a few days after being collected, there are usually 45 to 50 mature cercacariae which actively move about within the sporocyst. Measurements in the following description are from 10 living second-generation sporocysts.

Second generation sporocysts 3700 by 176. Anterior end with solid, tapered, active snout. Subterminal birth pore 56 to 72 from anterior end. Posterior end broadly rounded. Flagellets, 3 to 5 long, arising from papillae, scattered over entire surface. Transverse bands occasionally present posterior to birth pore.

Comparison: Other cyathocotylids with sporocysts that have a subterminal birth pore are *Prohemistomum vivax* (Sonsino, 1892) as described by Looss (1896), *Paracoenogonimus szidati* as described by Anderson and Cable (1950), *Prohemistomum chandleri* described by Vernberg (1952), *C. kasenyi* and *C. schoutedeni* described by Fain (1953), and *C. caribbea LI* described by Cable (1956).

Cercaria tatei was described by Johnston and Angel (1940) as being produced by sporocysts with muscle bands. While many fresh sporocysts of *M. kentuckiensis* exhibited these transverse bands, many others did not. The bands are not apparent in fixed material or in living material under pressure of a cover slip. For these reasons the bands may be of no importance in the identification of species. Cable (1938) and Vernberg (1952) did not report them for *C. kentuckiensis.*

The Cercaria (figs. 3, 4, 5)

Living cercariae of *M. kentuckiensis* were examined unstained or stained with neutral red, nile blue sulfate, or Bismarck brown. Examination of living cercariae in tap water, various physiological salt solutions, or aqueous polyvinyl alcohol was facilitated by flattening the cercariae under pressure of the cover slip. Fixed cercariae, used for measurements, were prepared by adding water containing the cercariae to an equal volume of boiling 10 percent formalin. Flagellet measurements were from living cercariae.

Description of the cercaria: Longifurcate, pharyngeate distomes with characteristics of the Vivax subgroup of Sewell, 1922, as emended by Dubois (1951), and of the subsubgroup (e) of Dubois (1951). Body flattened dorsoventrally, concave ventrally, oval to pyriform; 240 to 289 (262) by 117 to 154 (138). Body spinose, except for ventral surface in region of future tribocytic organ behind ventral sucker. Spination heaviest at anterior end, spines being thorn-shaped, and at posterior end, here spines being long and narrow. Brownish tinge to lateral and posterior portions of body. Fifteen flagellets, approximately 4 microns long, visible along each lateral margin of body; none observed on surfaces.

Tail inserted on dorsal side of body; tail-stem 323 to 440 (386) by 54 to 69 (60). Furcae flattened laterally; 258 to 316 (283) in length; provided with dorsal and ventral fin-folds, con-

tinuous around tips, extending 30 microns beyond tips of furcae. Ventral fin-folds extending approximately two-thirds length of furcae, dorsal fin-fold slightly shorter. Surface of tail-stem and proximal one-fourth of furcae finely spinose.

Lateral edges of tail-stem with flagellets, approximately 18 to 23 microns long, 40 on each side. Anterior 20 on each side rather far apart, occupying two-thirds of tail-stem, alternating dorsally and ventrally along margin of tail-stem. Posterior 20 arranged in pairs on each side. One of each pair ventral, the other dorsal. Each flagellet arising from granule in cuticle. Other flagellets located on dorsal and ventral surfaces of tail-stem, but pattern not determined.

Single flagellet located on posterior surface near tip of each furca; approximately half length of those on tail-stem; located 5 to 10 microns from tips of furcae or 35 to 40 microns from tips of furcal fin-folds; visible only on furcae viewed from either a dorsal or ventral position. Other smaller flagellets, each emerging from small papilla, located on posterior surface of furcae where latter joins the fin-folds (fig. 4); observed only on furcae flattened by cover slip pressure exposing flat surfaces of furcae; number variable, 16 o 20 on dorsal edge and 16 to 22 on ventral edge of each furca. Papillae at base of flagellets approximately 1.5 microns in diameter; flagellets approximately 5.5 microns long. Papillae not evenly distributed along their course. Second papillae and their flagellets smaller than others, located more centrally on furcae.

Protrusible oral sucker 61 to 68 (64) by 47 to 48 (47), containing 8 gland cells, opening anteriorly through individual ducts. In stained sections, granular contents of ducts eosinophilic, gland not. Pre-pharynx short, length 9 or less; muscular pharynx 220 to 243 (233) by 196 to 212 (202). Esophagus 20 microns long; intestinal ceca voluminous, sinuous, extending to posterior end of body. Walls of ceca often appearing crenated. Ceca containing irregularly shaped, freefloating, plastic bodies exhibiting birefringence when viewed with polarized light. Acetabulum 209 by 220, located 0.60 to 0.65 (0.62) of length from anteror end of body. Irregularly shaped mass of cells behind acetabulum, presumably genital primordium.

Excretory system typical of cercariae in Vivax subgroup. Four trunks extending anteriorly from excretory bladder, two median ones fusing just anterior to acetabulum forming a single median trunk, latter bifurcating just posterior to esophagus into right and left cross commissures connecting with lateral trunks. Arising from each commissural vessel an anteriorly directed vessel, unbranched or divided into anterior median and anterior lateral branches. Anterior median branches ending blindly at level of pharynx or uniting with each other across pharynx (fig. 5). All vessels mentioned above containing refractile granules, about 1.5 microns in diameter, except paired median trunks and lateral trunks. Anterior and posterior secondary collecting tubes joining to form short primary collecting tube on each side entering lateral trunk. Each secondary collecting tubule receiving capillaries of three groups of three flame cells resulting in flame cell formula therefore, of $2[(3+3+3)\ (3+3+(3))]=36$, last 3 on each side being located in tail-stem.

Furcal branches of caudal excretory tube emptying by pores on posterior surface of furcae just proximal to their tips. Tail-stem portion of caudal excretory tube moving freely in tail-stem, surrounded by matrix of cells with large nuclei to which flame cells and their capillaries attach. Caudal bodies with conspicuous nuclei on each side of caudal tube either in cercariae within sporocysts or in emerging cercariae; less obvious or absent in older cercariae.

Ill-defined glands in body lateral to pharynx, probably emptying to outside by ducts through oral sucker. In stained sections at least 22 ducts (11 pairs) observed, 8 of these from gland cells in oral sucker, remaining 14 probably pertaining to glands lateral to pharynx. Numerous unicellular glands, each opening immediately upon surface of worm, scattered over cercarial body. Some of latter glands still present in metacercariae encysted for 90 days.

Nile blue sulfate and neutral red taken up most strongly by concretions in intestinal ceca.

Comparisons: The cercaria, as described above, differs in only a few points from the description given by Cable (1938); however, additional morphological features have been described which perhaps will permit it to be readily identified and separated from other cyathocotylid cercariae.

The specimens measured by me were killed in hot 10 percent formalin, while those of Cable were measured alive under slight coverglass pressure. The measurements given by Cable fall within the ranges given here for fixed specimens. Since fixation generally shrinks specimens, it can be concluded that my cercariae are larger than Cable's, a fact substantiated by measuring some cercariae by the method of Cable (1938). A comparison of measurements of fixed and living cercariae from the same snail reveals that the fixed ones are 20 to 25 percent smaller.

Cable did not report the flame cell formula, but did report 14 flame cells in the body, while 15 were observed by Vernberg (1952). Cable reported the tail-stem to be without spines. However, Anderson (1944) and I have observed these on the tail-stem of the cercaria of *M. kentuckiensis.* While I have reported 15 flagellets on the margins of the body, Cable reported only "about five." These are viewed with great difficulty; however, the largest number seen was 15, an observation confirmed many times. The pores of the excretory tubules in the furcae were not seen by Cable who believed them to be absent. Other additional features described by me are limited mainly to the number and distribution of flagellets on the tailstem and furcae. Their arrangement in rather definite patterns is probably of value in species identification of cyathocotylid cercariae as well as other strigeate cercariae.

Other adequtely described cyathocotylid cercariae which closely resemble the cercaria of *M. kentuckiensis* are *Cercaria tatei* Johnston and Angel, 1940, and the cercariae of *Szidatia joyeuxi* and *Prohemistomum chandleri.*

Cercaria tatei differs in not having concretions in the intestinal ceca and in lacking flagellets on the body, tail-stem, and furcae.

The cercaria of *Szidatia joyeuxi* differs in the absence of anterior vessels from the commissural vessels; in the absence of refractile granules in the anterior portions of the excretory vessels; and in the absence of flagellets anywhere on the cercaria. The flame cell pattern has never been completely investigated for the cercaria of *Szidatia joyeuxi.* Of four descriptions of the cercaria, two described and illustrated concretions in the intestinal ceca, one described the intestinal ceca as being empty, while the fourth did not discuss them, but contained an illustration of the cercaria devoid of intestinal concretions.

Vernberg (1952) described the cercaria of *Prohemistomum chandleri* as being almost identical to *C. kentuckiensis.* She stated that the only differences are that *P. chandleri* is larger, lacks caudal bodies, and differs in development of the anterior vessel which arises from each commissural vessel. According to her, this vessel is always undivided in *P. chandleri,* while in *C. kentuckiensis* it is divided anteriorly into a lateral and a median branch. The median branch may or may not unite with the one on the opposite side (fig. 5). I have found cercariae from a single snail, some of which had the characteristic unbranched vessel of *P. chandleri,* while others had the characteristic branched vessel of *C. kentuckiensis* as described by Vernberg (1952). A second difference between these two cercariae observed by Vernberg is the difference in size. Vernberg recovered *C. kentuckiensis* from *Goniobasis livescens* collected from Clifty Creek in southern Indiana; while the cercariae of *P. chandleri* were recovered from *Pleurocera acuta* collected in northern Indiana. Cercariae of *M. kentuckiensis* from *G. livescens* collected by me from Clifty Creek in Indiana were also smaller than those obtained from snails collected in central Ohio, but were morphologically similar to the latter in all other respects. I believe that cercariae of a single species obtained from different species of snails from widely separated streams might vary in size as much as has been described for these two cercariae recovered by Vernberg. A third difference observed by Vernberg between *Cercaria kentuckiensis* and the cercaria of *P. chandleri* was in the degree of development of the caudal bodies. I believe their prominence to depend upon the age of the cercaria, becoming less prominent with time after emergence. Hoffman (1958) has observed this to be true of the caudal

bodies of the cercaria of *Ornithodiplostomum ptychocheilus.*

Since Vernberg did not show flagellets on the tail-stem in her drawing of the cercaria of *P. chandleri* (nor on her drawing of *C. kentuckiensis*), and pointed out that only differences between this cercaria and *C. kentuckiensis* were the three mentioned, the presence of these flagellets on the cercaria of *P. chandleri* remains in doubt.

Since the three differences pointed out by Vernberg between *Cercaria kentuckiensis* and the cercaria of *P. chandleri* are probably only variations within a single species, the latter is probably a synonym of the cercaria of *M. kentuckiensis* (i.e. *Cercaria kentuckiensis*).

Activity of the cercaria: The cercaria of *M. kentuckiensis* responds to shadowing by rapidly swimming tail-first in a series of jerks. The direction of tail flexure is apparently due to chance, being observed as many times to the right as left.

Cercariae exhibit a definite photonegative response when placed in a room darkened except for the light of a 60-watt bulb located about 6 feet to one side of the dish.

The number of cercariae emerging from one snail was observed at 8-hour intervals over a period of 80 hours. The most unproductive 8-hour period is that between 8:00 a. m. and 4:00 p. m., the period of continuous light. The greatest number of cercariae emerged from 4:00 p. m. to 12:00 p. m. A greater number during this period is to be expected since it follows a period during which, presumably, cercariae are still being produced.

Cercariae did not emerge from infected snails collected during the winter months until 14 days after they were brought into the laboratory. During the warmer months of the year they emerge the first or second day after collection.

The Metacercaria (fig. 6)

Measurements of the metacercaria were from 12 worms 90 days old, released from their cysts, fixed in hot 10 percent formalin, stained, and mounted. These measurements are 10 to 34 percent smaller than those of living metacercaria under light coverslip pressure. Measurements of the cysts were made while the cysts of living metacercariae were floating free in cold Ringer's solution with no coverslip over them.

Body 190 to 344 (270) by 165 to 219 (180); oral sucker 30 to 37 (34) by 32 to 45 (34); pharynx 24 to 34 (30) by 21 to 29 (24); acetabulum 21 to 26 (23) by 21 to 26 (24); tribocytic organ 40 to 58 (47) by 35 to 48 (43); genital primordium 27 to 42 (34) by 19 to 27 (24). Acetabulum located 0.43 to 0.57 (0.51) from anterior end. Flame cell number still 15 pairs in metacercaria 90 days old. Bladder and enlarged excretory tubules black by transmitted light due to refractile concretions within them.

Inner hyaline cyst of cercarial origin, at 3 hours, 155 by 140; at 8 days, 213 to 267 (250) by 206 to 220 (214); at 16 days, 264 to 311 (283) by 218 to 284 (286); after 90 days, varying considerably in dimensions but most only slightly larger than 16-day-old cysts.

Outer yellow cyst of host origin loosely constructed, without melanophores, considerably thicker in older metacercaria.

Comparison: The metacercaria of *Szidatia joyeuxi* has been sufficiently described to make a comparison with the present form. The dimensions of it are about twice those of the metacercaria of *M. kentuckiensis.* The most conspicuous difference between them is in the relative dimensions of oral sucker and pharynx. In *S. joyeuxi* the oral sucker is 50 to 100 percent larger than the pharynx, while in *M. kentuckiensis* the oral sucker is never more than 20 percent larger than the pharynx.

The Adult (fig. 8)

Measurements in the following description are from 5 of 13 adult specimens obtained from the intestine of a chick which had been fed metacercariae 10 days previously. The metacercariae were obtained from a specimen of *Lebistes reticulatus* experimentally infected with cercariae 29 days previous to being fed to the chick. The presence of trematode eggs in the feces of the chick on the seventh day indicated that at least one of the worms had been an egg-laying adult for about 3 days.

Mesostephanus kentuckiensis (Cable, 1935) n. comb.

Specific diagnosis: With the characters of the genus *Mesostephanus*. Body pyriform, spinose to level of ovary, divided into two regions; anterior region yellow-orange in life, flat and wide; posterior region shorter, narrow cylindrical, and truncated. Total length, 1,078 to 1,545 (1,281); maximum width at level of acetabulum 350 to 522 (400). Oral sucker subterminal 58 to 70 (64) by 61 to 67 (64). No prepharynx. Muscular pharynx 70 to 86 (79) by 68 to 89 (79); with cavity flattened laterally. Esophagus 40 to 88 (65) in length, frequently contracted. Intestinal ceca sinuous to level of acetabulum, then converging slightly before extending to posterior border of posterior testis. Acetabulum feebly developed, located anterior to tribocytic organ and vitellaria, 28 to 44 (33) by 32 to 47 (41), located 0.23 to 0.34 in the length of the body (av. 0.30). Tribocytic organ well developed, with median slit, glandular, 273 to 376 (328) by 174 to 248 (207).

Testes tandem, oblong or subspherical, in posteror half of body, median or slightly to right; linear extent 277 to 363 (328); anterior testis slightly larger, 144 to 184 (147) by 102 to 160 (127). Cirrus sac large, cylindrical, anterior end extending to posterior level of vitellaria, 360 to 563 (453) by 85 to 104 (93). Convoluted seminal vesicle occupying anterior one-fourth of cirrus sac, glandular pars prostatica the middle one-half or more, the cylindrical cirrus the last one-fourth or less. Genital atrium small, genital pore terminal. Ovary between and to right or left of testes, measuring 56 to 104 (80) by 80 to 96 (85). Mehlis' gland diffuse, medial, and posterior to ovary. Laurer's canal not observed. Uterus with ascending limb and anterior portion of descending limb occupying area between vitellaria, posterior portion of descending limb parallel or ventral to cirrus sac, terminated by vaginal sphincter before entering genital atrium. Vaginal sphincter 56 in diameter, 14 to 19 in thickness. Eggs large, few (1 to 4), operaculate, yellow, measuring 95 to 114 (103) by 63 to 75 (67). Vitellaria composed of large vitelline follicles, extending from anterior border of tribocytic organ to anterior border of posterior testis; ventral and lateral to genital apparatus, follicles disposed in two rows, their anterior portions being confined to the anterior half of tribocytic organ. Linear extent of follicles 311 to 501 (424).

Host (Experimental): *Gallus domesticus*

Site of infection: Small intestine

Hypotypes: U. S. National Museum Helminthological Collection No. 38384.

Comparison: *M. kentuckiensis* can easily be distinguished from other species of *Mesostephanus* by the large pharynx, which is always longer and wider than the oral sucker in *M. kentuckiensis*. It is also separated clearly by the location of the acetabulum, 0.23 to 0.34 of the length from the anterior end of the body. In other species it is never less than 0.37. *M. kentuckiensis* is longer in comparison to width than any other species except *M. cubaensis* as described by Dubois (1951). In life, the forebody of *M. kentuckiensis* is a bright yellow-orange, a condition not reported for other species of the genus.

Chandler and Rausch (1947) reported orange color as occurring in the vitellaria of *Neogogatea bubonis*. The type specimens of this species were examined as also were the type specimens of *Neogogatea pandionis* and *Mesostephanus longisaccus*. All three of these species originally had been described as lacking an acetabulum. Dubois (1951) had reported finding an acetabulum on specimens of *M. longisaccus*, a fact confirmed by me. In addition, I find an acetabulum on both species of *Neogogatea*. These worms are distinct from *M. kentuckiensis* in many respects.

DISCUSSION

The great number of species assigned incorrectly to one or the other of the two genera, *Prohemistomum* and *Mesostephanus,* testifies to their similarity. They were clearly separated by Dubois (1951), who pointed out distinct differences such as the length in relation to the width of the body, the location of the acetabulum in the body, the length of the body in relation to the diameter of the vitelline crown, and the nature of the termination of the uterus. In *Prohemistomum* the uterus ends in a metraterm while in *Mesostephanus* it ends with a vaginal sphincter. With regard to the location of the acetabulum in the body, *M. kentuckiensis* shows a resemblance to those worms in the genus *Prohemistomum.* The acetabulum is located between 0.23 to 0.34 of the body; while in all other species of *Mesostephanus* it is between 0.42 to 0.58. In species of the genus *Prohemistomum* it is between 0.25 to 0.40. The location of the acetabulum in *M. kentuckiensis* indicates that the position of this organ in the worms of these two genera is not of generic importance. Dubois (1953) states that the ovary is generally to the left of the median plane in *Prohemistomum,* except for *P. vivax,* and to the right of the median line in *Mesostephanus,* except for *M. appendiculatus.* Examination of the adults of *M. kentuckiensis* revealed that the ovary is on the right as often as on the left side of the median plane. As pointed out earlier, the cercaria of *Prohemistomum chandleri,* as described by Vernberg (1952), is similar to the cercaria of *M. kentuckiensis.* Dubois (1953) doubts the generic attribution of *Prohemistomum chandleri* upon the basis that vitelline glands had not yet developed and that the acetabulum, as illustrated, is 0.53 of the length from the anterior end of the body, the latter being a character of the genus *Mesostephanus.* In an individual of *M. kentuckiensis* recovered 3 days after feeding, the same age as the worms recovered by Vernberg, the acetabulum is 0.49 of the length from the anterior end of the worm. The great similarity of the immature worms as well as the cercariae of these two flukes again suggests that *Prohemistomum chandleri* is a synonym of *Mesostephanus kentuckiensis.*

Since the worms recovered by Vernberg from the snake after 3 days were developed little beyond the metacercarial state, a comparison of adults of these two species is impossible.

The genus *Mesostephanus,* erected by Lutz (1935) and redefined by Dubois (1951), now contains the following eight species: *M. fajardensis* (Price, 1934) Lutz, 1935 (Type) (Synonyms: *Prohemistomum fajardensis* Price, 1934, and *Mesostephanus prolificus* Lutz, 1935); *M. appendiculatoides* (Price, 1934) Lutz, 1935 (Synonym: *Prohemistomum appendiculatoides* Price, 1934); *M. appendiculatus* (Ciurea, 1916) Lutz, (1935 (Synonym: *Prohemistomum appendiculatus* Ciurea, 1916); *M. cubaensis* Alegret, 1941; *M. haliasturus* Tubangui and Masilunga, 1941; *M. longisaccus* Chandler, 1950; *M. microbursa* Caballero, Grocott, and Zerecero, 1953; *M. kentuckiensis* (Cable, 1935) n. comb. (Synonyms: *Cercaria kentuckiensis* Cable, 1935, and possibly *Prohemistomum chandleri* Vernberg 1952).

Dubois (1951, 1953) is of the opinion that birds of the order Pelecaniformes are natural hosts of the adults of the genus *Mesostephanus.* Four of the seven previously known species of *Mesostephanus* occur in birds of the order Pelecaniformes. *M. haliasturus* was described by Tubangui and Masilungan (1941) from *Haliastur haliasturus* of the order *Falconiformes.* Two species, *M. appendi-*

culatus and *M. longisaccus*, are known to occur naturally only in dogs. Dubois (1951) refers to these two as secondary or erratic parasites in the dog.

Restricting species of the genus *Mesostephanus* to birds of the order Pelecaniformes would implicate either the white pelican, *Pelecanus erythrorhynchos*, or the double-crested cormorant, *Phalacrocorax auritus*, as the possible natural definitive host of *M. kentuckiensis*. Borror (1950) lists the former as a rare transient in Ohio, the latter as a rare to common spring and fall transient. *Phalacrocorax auritus* appears the most available possibility as the host. This species has been reported as the host of *Mesostephanus cubaensis*.

Even if species of *Mesostephanus* are natural parasites of birds of the order *Pelecaniformes*, one might occasionally appear in a bird of another order, especially in view of the apparent lack of host specificity as shown by the appearance of two species in dogs.

SUMMARY

The life history of *Mesostephanus kentuckiensis* (Cable, 1935) n. comb. is elucidated. The cercaria, *Cercaria kentuckiensis*, is redescribed and the daughter sporocysts, metacercaria, and adult are described.

Cercariae, recovered from *Goniobasis livescens*, were found to encyst and develop experimentally as metacercariae in laboratory-raised fish (*Lebistes reticulatus*) and amphibia (*Rana clamitans, R. catesbeiana, R. pipiens*). Ten species of fish and two species of amphibia were found to be naturally infected with the metacercariae.

Metacercaria from natural and experimental infections yielded, when fed to chicks, adults which were identical. Eggs of *M. kentuckiensis* first appeared in the feces of experimentally fed chicks after 7 days. In the chick, the life span of the adult trematode is between 14 and 17 days.

The possible synonymy of *M. kentuckiensis* and *Prohemistomum chandleri* based upon the similarity of their cercariae and immature adults is discussed.

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EXPLANATION OF PLATES

PLATE I

Figure 1. The egg of *Mesostephanus kentuckiensis*.
Figure 2. Daughter sporocyst of *M. kentuckiensis* containing germ balls and cercariae in various stages of development.
Figure 3. The cercaria of *M. kentuckiensis*. Dorsal view.
Figure 4. View of posterior surface of furca showing furcal flagellets.
Figure 5. Variations which occur in the canals anterior to the median and lateral excretory trunks.

PLATE II

(Camera lucida drawings)

Figure 6. The metacercaria of *M. kentuckiensis*. Ventral view.
Figure 7. Immature worm after 4 days in the chick. Ventral view.
Figure 8. The adult of *M. kentuckiensis*. Dorsal view.

PLATE I

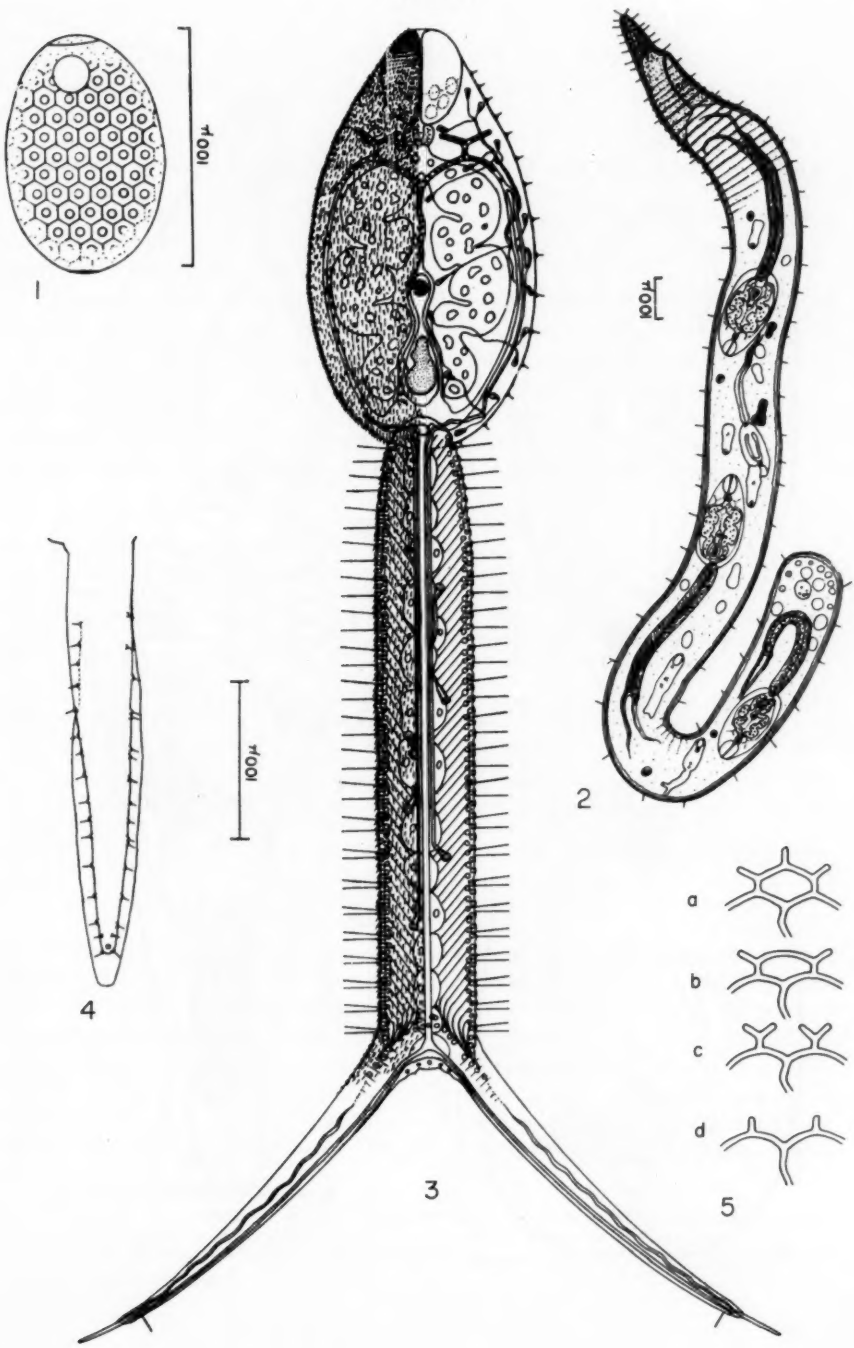
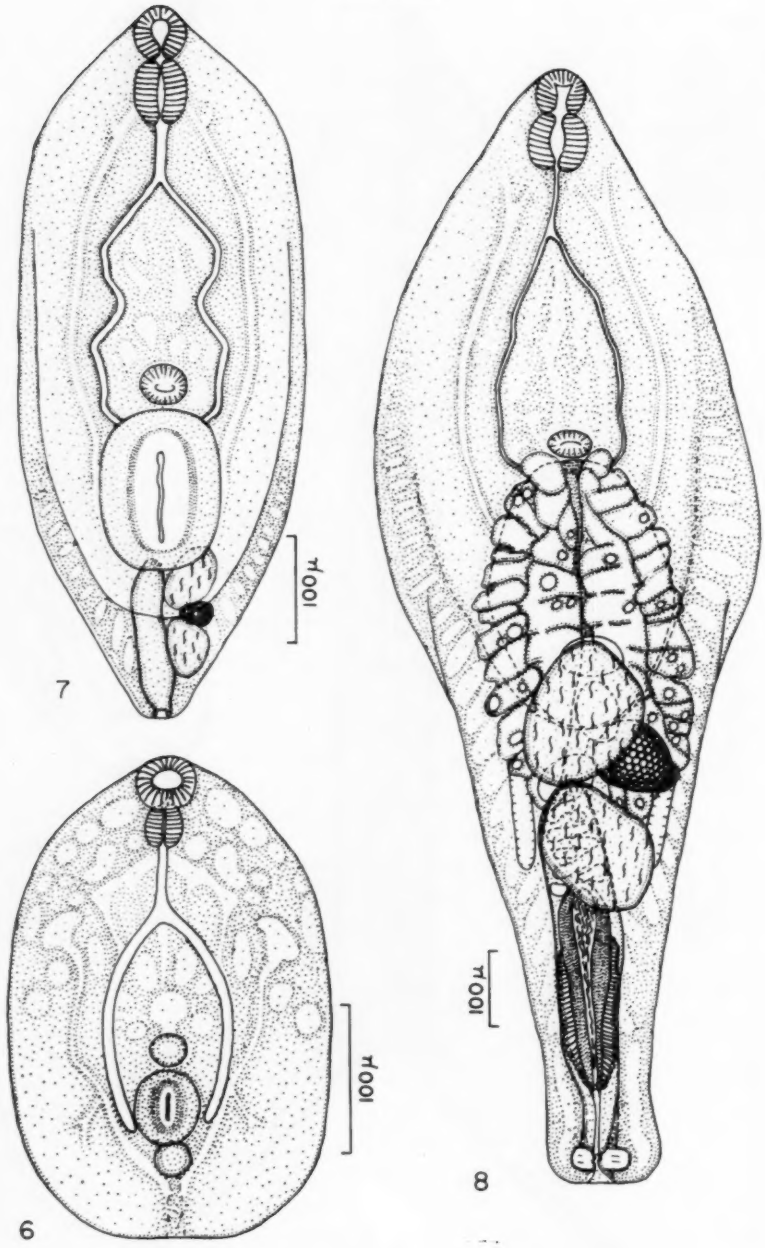


PLATE II



STUDIES ON THE LIFE HISTORY OF *ECHINOSTOMA NUDICAUDATUM* N. SP. (ECHINOSTOMATIDAE: TREMATODA)*

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There are apparently 117 described species in the genus *Echinostoma*. More or less complete life cycles are known for only three of these, namely, *E. revolutum*, *E. macrorchis*, and *E. lindoensis*.

The author studied 3,000 *Limnaea stagnalis* (L.) collected from Edgbaston Pool, an artificial lake near the University of Birmingham, Birmingham, England, for cercarial infection. Birds in this area could not be examined for adult infection, since Edgbaston Pool is in a wildlife sanctuary and the fauna is highly protected. Two thousand snails were found infected with cercariae of *E. nudicaudatum*. This wealth of material enabled the author to conduct the experimental studies on the various stages in the life history of this trematode reported in this paper.

MATERIALS AND METHODS

Experimentally clean stocks of *Lymnaea stagnalis* were established in the laboratory from eggs collected from Edgbaston Pool and the snails were maintained at a temperature of 19 to 24 C on a diet of lettuce. Experimental definitive hosts used were mice, ducks and pigeons. Ducks were obtained from commercial dealers as 3-day-old ducklings. Pigeons also were obtained from dealers. The mice were from stock maintained in the Department Mammal House. To avoid contamination, the pigeons and ducks were fed on commercial chicken mash and pigeon mixture.

Metacercariae were introduced orally into mice, ducks and pigeons with the aid of an ordinary medicinal pipette. Daily fecal examinations of all experimental definitive hosts were done for 1 week before the commencement of feeding experiments. No trematode eggs were found, but nematode eggs and coccidian cysts were sometimes present.

Vital stains methylene blue, nile blue sulphate, and neutral red were employed for study of larval stages. Adult trematodes required for whole mounts and sections were fixed by squirting into hot (60 C) Gilson's fluid. Paraffin sections were cut in various planes at 6 to 8 microns and stained with Mason's trichrome or celestine blue followed by picro-acid fuchsin.

Miracidium (Figs. 1 and 2)

(All measurements in millimeters)

The mature miracidium emerges from the egg 3 weeks after development at room temperature, 19 to 24 C. The free swimming miracidium measures 0.072 to 0.164 by 0.036 to 0.066. A collar-like transverse band encircles the body at the level of the first and second tiers of epidermal plates. Anteriorly the body bears a retractile apical papilla 0.014 to 0.022 by 0.004 to 0.006. Projecting from the free end of the papilla, there are delicate hair-like processes longer than the cilia and varying in number from 5 to 7. The body, with exception of the apical papilla, is covered with rows of cilia, 0.012 to 0.016 long. The cilia towards the anterior end are shorter

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than those more posterior, measuring 0.009 to 0.011. Because the successive tiers of the epidermal plates are separated by wide gaps, the distribution of the cilia is interrupted.

There are four tiers of epidermal plates (fig. 2) consisting of, respectively, 6, 6, 4 and 2 (= 18) plates from anterior to posterior end. Those in the first tier measure 0.014 to 0.024 by 0.008 to 0.019; in the second, 0.022 to 0.036 by 0.016 to 0.017; in the third, 0.028 to 0.048 by 0.027 to 0.032; and in the last tier, 0.017 to 0.028 by 0.022 to 0.028. In the anteriormost row, two of the plates are dorsal, two ventral, and two lateral; in the second row, three are dorsal and three ventral. In the third row, two are dorsolateral and two ventrolateral. The plates of the fourth and last tier are one dorsal and one ventral, overlying each other; in some of the active specimens they may appear to be slightly lateral in position.

A pair of lateral processes (fig. 1) are present, one on each side of the body in the groove between the first and the second tiers of the epidermal plates; each of these processes consists of a proximal narrow stalk and a dilated bulbous distal portion. No organs or internal connections appear to be associated with them. Immediately in front of each lateral process is a hair-like prolongation, stiffer than the process itself. These lateral processes are flexible but immobile. The distal dilation of the posterior lateral process may be markedly constricted off from its narrow stalk.

On each side of the body at the base of the apical papilla and slightly ventral to it is a short protuberance, which bears no connection with any other structures.

A pair of almost spherical dark brown eye spots are present posterior to the interspace between the first and the second tiers of the epidermal plates; usually these are separate from each other, but in some specimens they are contiguous or partially overlapping. Each of the eye-spots is furnished with a granular pigmented area.

The central nervous system is a quadrangular fibrous mass, located dorsally, in the median region of the second tier of the epidermal plates. It is surrounded by numerous nuclei, probably the nuclei of the nerve cells.

At the extreme anterior tip of the apical papilla is a small opening which seems to communicate with a clear neck portion (duct) of a flask-shaped apical gland, filled at its dilated basal portion with a coarsely granular material. Four or five large spherical nuclei are grouped in this posterior portion. The granular contents have been observed to discharge through its opening, similar to the discharge of the penetration gland contents of cercariae.

Ventral and lateral to the apical gland and sometimes extending considerably posterior to it is another pair of glands variously known as "cephalic glands" (Cort, 1919) or "penetration glands" (H. F. Price, 1931). These glands are flask-shaped and open externally onto the lateral sides of the body near the base of the apical papilla. The granular contents of these glands are much finer than those of the adjacent apical gland. Each gland has a large spherical nucleus at its base.

The excretory system has two flame cells, one anterior and one posterior to the equator of the body; the anterior is ventral and the posterior dorsal. Their capillaries open exteriorly, slightly dorsally, between the third and the fourth tiers of the epidermal plates. The capillaries may dilate slightly at the points where they empty into external pores.

A single multicellular germ ball is located near the posterior end of the miracidium, within the region bounded by the fourth tier of the epidermal plates. Posterior to the germ ball there may be six or more uninucleate germ cells, some of which extend as far anterior as the second tier of the epidermal plates.

Redia (Figs. 3 and 4)

(All measurements in millimeters)

The rediae exhibit great diversity of color, shape, and size. Three kinds of rediae were found in the digestive glands of the snails: (a) mother rediae containing only daughter rediae; (b) daughter rediae containing only cercariae in different developmental stages; (c) rediae containing both daughter rediae and cercariae. Of these various redial forms, the second form, i.e. daughter containing only cercariae, is most common and is found throughout the year, often in such large numbers that in a single host specimen the whole of the digestive gland becomes an intertwined mass of rediae; the mantle cavity and other organs of the snail may also be invaded. The first redial form, i.e. rediae containing only daughter rediae, is most abundant during autumn, but never as abundant as rediae containing cercariae. The rediae containing both daughter rediae and cercariae are the least common and have been observed only on two occasions by the author. However, Wesenberg-Lund (1934) encountered this stage "very often" in redia of *C. echinata.*

Johnson (1920), F. G. Rees (1940), Churchill (1951), Wu (1951) and Najarian (1954) believe there exist only two redial generations: the mother, or first generation producing only daughter rediae; and the daughter or second-generation rediae producing only cercariae.

Mathias (1925) believed that the daughter rediae (those produced by the original mother redia) could give rise either to cercariae or to further generation of rediae which in turn would eventually produce cercariae.

In the present investigation all attempts to infect the laboratory-bred snails with miracidia of *E. nudicaudatum* were unsuccessful. The first generation mother rediae could not be identified. However, knowledge gathered from natural infections indicates that there are at least four generations: the first generation or mother rediae, the second generation or daughter rediae, and the third generation rediae, which simultaneously produce fourth generation rediae as well as cercariae. It is not certain whether the fourth generation rediae produce cercariae or cercariae plus another generation of rediae, since in none of the fourth generation rediae, present inside the third generation rediae together with the developing cercariae, had germ balls sufficiently differentiated to distinguish between rediae and cercariae.

The immature rediae (fig. 3) measuring 0.376 to 0.429 by 0.056 to 0.08 are unpigmented, nearly cylindrical, and narrowed posteriorly. The mouth is located at the extreme anterior end, followed by a muscular pharynx, 0.057 to 0.068 by 0.051 to 0.055. The sacciform gut may extend as far posterior as the locomotory appendages, and contains no food material. These appendages arise from the ventrolateral aspect of the posterior part of the body. There is a conspicuous collar behind the pharynx. The distance of the collar from the anterior end of the body depends upon the state of contraction of the highly contractile precollar region.

The mature rediae containing either developing rediae or cercariae are light orange or sometimes yellow in color; the intestine contains yellow food material. The majority of these rediae are 0.8 by 0.2, with the pharyngeal diameter about 0.09;

some specimens may be as large as 4.0 by 0.52, with the pharynx measuring about 0.2.

There is a distinct birth pore 0.02 in diameter on one side of the body immediately behind the collar. In some specimens the birth pore may protrude from the surface. In 200 immature specimens studied the birth pore could not be traced; seemingly its development is correlated with the differentiation of the germ balls into either rediae or cercariae. The intestine, which in the immature rediae reaches the locomotory processes and occupies nearly the whole extent of the body cavity between the pharynx and the locomotory processes, does not grow and may shorten, so that in older specimens it extends just beyond the collar region.

Older rediae in which the cercarial or the redial supply is exhausted are almost motionless and develop "orange flecks" in the body wall. The intestine is clear of food material. The collar is considerably weakened and in some specimens is observable only with difficulty. The birth pore, which may be produced like a nipple in the mature forms, could not be seen in seven "spent" rediae. The locomotory processes may occasionally be atrophied.

There are two groups of flame cells on each side of the body, one located at the levels of the locomotory appendage and the other a short distance anterior to the locomotory appendage. Each excretory vesicle lies laterally at the anterior margin of the locomotory process and receives two excretory ducts, one from the anterior group of flame cells and one from the posterior group. The external openings of these vesicles were not observed.

The study of flame cells in larval trematodes is difficult especially in rediae because of the presence of germ balls containing developing flame cells; further, the number of flame cells in rediae may increase with age (Tubangui and Pasco, 1933). Repeated observations on the rediae of *E. nudicaudatum* indicate that there are 11 flame cells in the anterior group and 10 in the posterior group on each side, making a total of 42. Johnson described 18 flame cells in the posterior group and 17 in the anterior group in the redia of *E. revolutum*. Beaver could not find this arrangement, but in the redia of the same species noted four groups each containing nine flame cells.

Three general types of structural differences were observed in the rediae of *E. nudicaudatum:* (1) Abnormal rediae, due to the presence of other larval trematode species within the redia, e.g., post-cercarial stages of *Cotylurus brevis* (unpublished.); (2) Changes with aging, older rediae with degenerate locomotory processes and greatly distorted body (Similar loss of lateral appendages in older rediae of *Echinopanyphium recurvatum* was observed by Harper, 1929); (3) A developmental anomoly was observed in one redia, namely, the partial division of the posterior end of the body (In other respects it was normal).

Cercaria (Fig. 5)

(Measurements in millimeters are based on 20 living specimens)

Diagnosis: Echinostome cercariae. Body length 0.463 (0.361 to 0.643), width 0.250 (0.219 to 0.296); tail without a finfold; length 0.430 (0.341 to 0.566), width at base 0.081. Cuticular spines, about 0.004 long, on both sides of body in preacetabular region; in postacetabular part their distribution not ascertained. Collar, well developed; 37 spines in all: two sets of 5 corner spines, 3 oral and 2 aboral, two sets of 7 unalternating lateral spines, 13 spines of dorsal series disposed in two alternating rows. Flagellets present on body and tail, densely disrtibuted in proximal region of latter. Oral sucker subterminal to terminal, bordered on its anterior lip by 12 openings which are apertures of the ducts leading probably from penetration glands;

0.067 (0.063 to 0.075) diameter. Acetabulum well behind equatorial line of body, protrusible, acetabular orifice slightly "papillate;" 0.109 (0.095 to 0.117) diameter. Prepharynx inconspicuous, 0.014 to 0.015 long. Pharynx anteroposteriorly elongate, 0.033 by 0.022. Esophagus in its anterior part as broad or even broader than pharynx, gradually tapering posteriorly, bifurcating in front of acetabulum; intestinal ceca extending almost to posterior end of body, symmetrically deflected towards median line in postacetabular region; esophagus and ceca enclosing "refracile bodies." Cystogenous glands opaque, rather pyriform, with granular not "rod-shaped" contents. Two cerebral ganglia, one on either side of pharynx, joined by a dorsal commissure; a prominent lateral nerve running from each ganglion to posterior end of body. Excretory vesicle bipartite, opening exteriorly through a small excretory pore located dorsally at the junction of body with tail; each main lateral collecting tube running dorsally to anterolateral margin of acetabulum where after a short backward bend it continues anteriorly, at pharyngeal level forms a typical "echinostome loop," then pursuing a tortuous course posteriad as a secondary lateral tubule; this secondary lateral tubule lined with ciliary patches throughout its entire extent excepting a short distance posterior to pharynx, after forming another loop at level of excretory vesicle, continuing again anteriorly where it terminates in three flame cells; main lateral tubes in preacetabular region distended as much as 4 to 6 times their normal size to make room for a large number (more than 200) "refractile excretory granules;" caudal excretory duct running down about one-sixth of tail length and dividing into two oblique lateral branches, latter apparently ending blindly. Flame cells, more than 47 on each side of body; 10 persistently lying on each side of preacetabular region. Development in rediae. Host, *Limnaea stagnalis* (L.), in Edgbaston Pool, Birmingham. Encystment in various species of snails and tadpole, never encysting on vegetation or dead objects.

Light has no unusual effect on the cercariae of *E. nudicaudatum*. After emergence the cercariae swim actively near the bottom of the container for 6 to 10 hours, then sink to the bottom and creep along the substrate by use of the suckers. After 24 hours, the cercariae are exhausted although they may survive in ordinary tap water at room temperature for another 24 hours.

Related Species: In the literature nine echinostome cercariae are described which, like the cercaria of *E. nudicaudatum*, possess 37 collar spines and are without tail finfold. Cephalic spination is probably the most reliable feature in determining the species of echinostome cercariae. To this may be added the granular or "rod-shaped" condition of the cystogenous gland cells, the importance of which has already been pointed out by Johnston and Angel (1941) and Sandground and Bonne (1940). The identification of the number and arrangement of the flame cells is also an important characteristic for the recognition of different species, but in practice this may be difficult. Beaver in his studies on *E. revolutum* was in doubt about the exact number of flame cells in the cercariae but was confident of his assessment of the number and arrangement of the collar spines.

Cercaria echinata Siebold, according to Brown (1926), Wesenberg-Lund (1934), and the cercaria of *Echinoparyphium aconiatum* Dietz, 1909, as described by Dubois (1929), each have a group of four corner spines. In the cercaria of *E. nudicaudatum* each of the corner groups has five spines. The contents of the cystogenous gland cells of *C. echinata* are "short rod-like bodies" (Wesenberg-Lund, p. 54), whereas the contents of the corresponding glands in the cercaria of *E. nudicaudatum* are roughly spherical granules. Beaver expressed doubt that *Cercaria echinata* was distinct from the cercaria of *Echinoparyphium aconiatum*.

Cercaria equispinosa, according to Brown (1926), differs from *C. echinata* "in size only," and is, therefore, a distinct species from the cercariae of *E. nudicaudatum*. *Cercaria rebstocki* (McCoy) as described by Byrd and Reiber (1940) is readily distinguished from cercaria of *E. nudicaudatum* by the possession of "rod-like" contents in the cystogenous glands.

Tubangui (1932) made no special reference to a finfold in the cercaria of *E.*

revolutum, but E. L. Miller (1936), Beaver (1937), and Johnston and Angel (1941) have observed a finfold to be present in this cercaria. Thus, it seems most likely that the cercaria of *E. revolutum* possesses a finfold, whereas the cercaria of *E. nudicaudatum* has no finfold.

A cercaria described by Sandosham (1953) as *E. revolutum* with no tail finfold resembles closely the cercaria of *E. nudicaudatum,* except that the caudal branch of the excretory system in the former (after giving rise to two lateral branches) is continued to the posterior end of the tail, while in *E. nudicaudatum* there is no continuation of the caudal excretory branch after it has divided into two lateral branches. Moreover, the cercaria of Sandosham develops into the adult, *E. revolutum,* a distinct species from *E. nudicaudatum.*

Cercaria helvetica XXII, XXIII, and *XXV* Dubois (1929) each has a pattern of collar spines almost identical with that of *E. nudicaudatum;* these four species are almost indistinguishable from one another on the basis of this character. However, *C. helvetica XXII* is nearly twice as large as *E. nudicaudatum;* the diameter of the oral and the ventral suckers in *C. helvetica XXIII* is considerably larger than the same structures in the cercaria of *E. nudicaudatum; C. helvetica XXV* is as yet incompletely described, but from available data appears to resemble the cercaria of *E. nudicaudatum* so closely that it may prove to be the same species.

Metacercaria

To be infective the cercariae of *Echinostoma nudicaudatum* must leave the first intermediate host (i.e., the particular specimen of *L. stagnalis* into which the miracidium entered) and spend a short free-living period before entering and encysting in another host. The second intermediate host may be of the same species as the first intermediate host, or it may be the same specimen from which the cercariae emerged, or a snail of a different species of *Limnaea,* or various species of *Planorbis, Paludina, Bithynia.* Encystment was also obtained in tadpoles. Repeated experiments with both laboratory-bred and naturally infected hosts indicate that the cercariae of *E. nudicaudatum* exhibit a marked preference for encystment in the renal organs.

The cysts are almost spherical, measuring 0.248 (0.232 to 0.304) in diameter.

Infection of Pigeons through Naturally Infected Limnaea stagnalis *(summary)*

Exp. 1. Pigeon 1 was fed thousands of cysts along with renal organ on 28 Aug. 56; on 30 Aug. 56, 31 immature flukes recovered from duodenum.

Exp. 2. Pigeon 2, given thousands of cysts on 3 June 56; on 6 Sept. 56 two flukes found in ileum about 30 cm posterior to stomach.

Exp. 3. Pigeon 3, thousands of cysts in renal organ on 3 Sept. 56; on 7 Sept. 56, 46 worms in anterior part of ileum each containing one or two eggs in uterus.

Exp. 4. Pigeon 4, over 2000 cysts on 17 Sept. 56; dissected on 25 Sept. 56, negative.

Exp. 5. Pigeon 5, over 2000 cysts on 17 Sept. 56; dissected on 26 Sept. 56, negative.

Exp. 6. Pigeon 6, over 3000 cysts on 25 Sept. 56; dissected on 1 Oct. 56, negative.

Exp. 7. Pigeon 7, over 3000 cysts on 25 Sept. 56; dissected on 9 Oct. 56, negative.

Exp. 8. Pigeon 8, 90 cysts on 17 Oct. 56; feces positive on 31 Oct. 56; dissected on 2 Nov. 56, five mature flukes in rectum.

Exp. 9. Pigeon 9, 90 cysts on 17 Oct. 56; positive on 31 Oct. 56; dissected on 5 Nov. 56, three worms in cloaca.

Exp. 10. Pigeon 10, 900 cysts on 31 Dec. 56; positive on 14 Jan. 57; dissected on 15 Jan. 57, 12 worms in cloaca.

Infection of pigeons through experimentally infected laboratory-bred L. stagnalis (*summary*)

Exp. 11. Pigeon 11, two renal organs with thousands of cysts on 22 Feb. 57; positive on 8 Mar. 57; eggs appeared continuously until 18 Mar. 57; dissected on the same date, negative.

Exp. 12. Pigeon 12, 12-month-old cysts on 18 Mar. 57; feces positive on 1 Apr. 57, became negative on 17 Apr. 57, dissected on the same date, no infection.

Exp. 13. Pigeon 13, more than 14-month-old cysts on 18 Mar. 57; positive on 1 Apr. 57; eggs continuously appeared til 4 Apr. 57 when a degenerating fluke was found in the feces; dissected on 5 Apr. 57, 3 decaying flukes in cloaca. This pigeon developed diarrhea on the same date, i.e., 1 Apr. 57, when the first fluke eggs appeared, and died 4 days later.

Exp. 14. Pigeon 14, more than 15-month-old cysts on 15 May 57; stool examined on alternate days until 3 June 57, but no eggs appeared.

Exp. 15. Pigeon 15, 4-day-old cysts on 20 May 57; stool negative; dissected on 7 June 57, negative.

Exp. 16. Pigeon 16, more than 2000 four-day-old cysts from four snails, on 20 May 57; stool negative; dissected on 27 June 57, negative. This pigeon died suddenly on 27 June 57.

Exp. 17. Pigeon 17, 350 five-day-old cysts on 3 June 57; positive on 17 June 57; dissected on 18 June 57, seven flukes in cloaca.

Exp. 18. Pigeon 18, 900 eight-day-old cysts on 23 June 57; positive on 7 July 57.

In addition to the pigeons, 6 mice and 8 ducks were used, but the results were always negative.

Conclusions

(1) Pigeons proved to be the most suitable laboratory definitive host of *E. nudicaudatum*. Of 18 pigeons fed experimentally, 11 became infected, 2 were not infected because immature metacercaria were fed; the remaining 5 attempts to infect were unsuccessful for reasons unknown.

(2) The minimum duration of the cyst in the snail host, before successful infection of the definitive host (pigeon 17) may occur, is 5 days (laboratory temperature 19 to 24 C). In the natural environment, where the temperature is relatively low, this period may be longer. Cysts 5 days old to more than 14 months old were found to be infective. It seems probable that in nature the cysts remain infective as long as their host remains alive.

(3) During the experiments with pigeons (Nos. 6, 7, 10, 11, 12, 13, 17, 18) it was observed that eggs first appeared in the feces of the host on the 15th day after the introduction of the cysts. As is shown in the experiment (No. 1) on the 3d day of the introduction of cysts, the immature flukes were liberated in the duodenal region; on the 4th day (Exp. 2) the flukes were scattered about 30 cm posterior to the stomach, while on the 5th day (exp. 3), they were found further down along the small intestine and each by this time had one or two eggs in the uterus.

(4) Observations on pigeons (11 and 12) indicate that the adult specimens of *E. nudicaudatum* remain in the definitive host 25 to 32 days. The first 15 days are spent in development, the last 10 to 17 days as an active egg-producing stage; ultimately the parasite is passed out of the body of the host.

(5) The site of natural infection in pigeons is the cloaca (exp. 7, 10, 13, 17); of 30 adults recovered in these experiments, 83 percent were from the cloaca, 17 percent from large intestine and none from the small intestine.

Adult (Figs. 6 to 12)

(Based on observations of 17 egg-discharging adults fixed in hot Gilson's fluid. Measurements in millimeters.)

Diagnosis: Genus *Echinostoma* Rudolphi, 1809, sensu Dietz (1909; 1910) emend. Mendheim (1943.) Body length 6.832 to 7.632; greatest width always in uterine region, 0.984 to 1.280; average ratio of breadth to length 1 : 6.1. Cuticle with transverse rows of spines, distribution of spination differing on dorsal and ventral surfaces of body; ventrally, spination extending posteriorly to half way between posterior testis and posteris extremity of body, often becoming more sparsely distributed, but never completely absent behind anterior testis; dorsally, confined to a region anterior to acetabulum, within this region, density of distribution usually decreasing from anterior to posterior. Head collar, 0.472 to 0.528 broad, wth a row of 37 spines, 0.060 (0.052 to 0.081) long, continuous dorsally but interrupted ventrally in pharyngeal region, and arranged in the (3+2)+7+(6+7)+7+(3+2) fashion; 5 corner spines on each side, with innermost oral spine the shortest and the lateral aboral spine of the corner group the longest. Seven unalternating spines on each side; 13 dorsal spines with 6 orals slightly shorter than 7 aboral (oral: aboral = 1 : 1.2 approx.); median spine of whole series of dorsal spines aboral in position. Oral sucker, 0.244 to 0.256 by 0.208 to 0.232; oral aperture, 0.088 to 0.104 by 0.104 to 0.136. Ventral sucker, 0.528 to 0.60 by 0.552 to 0.608, between a sixth to an eighth of the body length from anterior end, about two and a half times as large as oral sucker; cuticular lining of acetabular orifice thrown into indentations, latter more pronounced along the posterior border. Distance between two suckers 0.584 to 0.920. Prepharynx, 0.04 to 0.045 long, may not be seen in whole mounts, always observable in sectioned material. Pharynx, globular, 0.184 to 0.192 by 0.153 to 0.168, average ratio of its diameter to that of oral sucker, 1 : 1.4. Esophagus, broadest immediately behind pharynx, 0.28 to 0.36 long, bifurcating at a point about one-ninth of total body length from anterior extremity; intestinal ceca reaching almost to posterior end of body. Distance between posterior terminations of intestine and posterior end of body, 0.152 to 0.272. Testes tandem, somewhat oval in shape, with entire margins in living and well fixed material, irregularly bordered in poorly fixed material but irregular borders not constituting lobing; anteroposterior dimensions of both testes greater than breadth; both testes occupying a position in first third of posterior half of body, anterior testis lying immediately behind equator of body; anterior testis, slightly smaller than posterior testis, 0.48 to 0.584 by 0.224 to 0.312, at a distance of 1.864 to 2.152 from acetabulum; posterior testis, 0.552 to 0.64 by 0.224 to 0.312, at a distance of 0.032 to 0.176 from anterior testis, and at a distance of 2.296 to 2.680 from posterior end of body. Intertesticular space usually one-third of length of anterior testis, distance from posterior testis to posterior end of body approximately one-third of total body length. Muscular cirrus sac, 0.288 to 0.352 by 0.168 to 0.232, somewhat pyriform, lying in position anterior to middle of acetabulum with its base slightly to one side, usually on left, of median sagittal plane of body; in living and some sectioned material entirely anterior to acetabulum; pars prostatica present. Ovary, 0.25 to 0.277 by 0.288 to 0.328, spheroidal, median, just in front of body equator, with a diameter of about 0.04 of body length, ratio of its diameter to length of oral sucker, pharynx and cirrus sac about 1 : 1, 1.5 : 1 and 1 : 1 respectively; separated from acetabulum by a distance of about one-fifth of total body length. Distance of ovary from anterior testis and acetabulum, 0.216 to 0.256, and 1.40 to 1.672. Vitelline glands never extending to equator of acetabulum.

Common genital pore, median or slightly to right of median line, immediately beneath esophageal bifurcation, at a distance of 0.048 to 0.08 from ventral sucker. Eggs, yellowish, ovoid, operculate end slightly narrowed; naturally laid eggs (sample of 50) measuring 0.104 (0.097 to 0.115) by 0.069 (0.067 to 0.072).

There have been conflicting statements in the literature concerning the cuticular spination of echinostomes. Hilario and Wharton (1917) wrote of *Euparyphium ilocanum* as follows: "the spines are developed and lost very early in the life of some worms or some may fail entirely to develop spines at any time." Beaver (1937) observed the presence of spination in *E. revolutum* "on the ventral side of the body in all individuals, but the larger specimens, 10 mm in length or more, may be without dorsal cuticular spines."

Cuticular spination of the adult echinostome may well be of taxonomic importance; however, its reliability may be destroyed by post-mortem changes which take place in long dead hosts and delaying proper fixation of living worms after recovery. E. W. Price (1931) and Beaver (1937) call attention to these factors and their effect on spination.

The author in this study recovered 25 dead young flukes from a pigeon dead for about 6.5 hours; 14 of these flukes had only small patches of cuticle intact; the remaining 11 had completely lost all cuticle and spines. This experience further emphasizes the meticulous care that must be exercised in recovery and preparation of specimens if the spination character is to be a reliable one.

Hilario and Wharton (1917) and Sandground and Bonne (1940) point out that collar spines are also lost under the conditions mentioned above.

It may be concluded then that while cuticular spination may well be of a taxonomic importance in echinostomes, its use in the past has been unreliable because insufficient attention has been paid to factors which may interfere with the state of preservation of the cuticle. Beaver further emphasized the need for a thorough investigation of the cuticular spination and added: "For just as it cannot be said that *E. revolutum* is either with or without dorsal cuticular spines without reservation, the same probably holds true for other species."

Accurate measurements are exceedingly difficult to make because collar spines may be in oblique planes; therefore, only large differences in size are likely to be of taxonomic importance.

The relative locations of the individual spines of the angle group are easily distorted during the handling of specimens in histological processes. Thus, frequently the first lateral spine of the unalternating series comes to occupy a position nearer to the five angle spines giving the appearance of "six" angle spines; further the innermost of the angle spines may occupy an aboral position, while normally it is located orally.

During the present study a special watch was kept for differences that might occur in the number of collar spines present in the cercaria and in the adult. A total of 27 living adults and 50 living cercariae were examined, in all cases the number of spines was constant at 37, in both adult and cercaria, the only variations were slight differences in position due to manipulation. These observations support Tubangui's (1932) general contention that "there exists a specific resemblance between echinostome cercariae and their adults with regards to the number of spines on their head collar," and disagree with the views of some workers who believe that the number of collar spines in the adult may differ from the number present in

the cercaria. Johnson (1920) described the cercaria of *E. revolutum* as having 43 collar spines whereas the adult has 37. Both Tubangui and Beaver suggested that Johnson observed the spination of another species of cercaria. On the other hand, Iwata and Tamura (1933) in the same species, *E. revolutum,* described the head crown usually with 37 spines, but the younger ones have only 35.

Reproductive system: Testes as previously described. The vasa efferentia (figs. 6, 9 and 10) after emerging from the testes run parallel and anterior entering directly the posterior end of the cirrus sac. The cirrus sac encloses a large coiled seminal vesicle and a terminal unspined cirrus; the latter organ may be protruded, beyond the genital pore, to a length slightly greater than that of the sac itself. The voluminous pars prostatica, more dilated than the ejaculatory duct, is not easily seen in whole mounts and is best identified in frontal sections.

The ovary is as described in diagnosis. The oviduct (figs. 11 and 12) arises from the posterior border of the ovary and soon gives off the Laurer's canal which curves to open on the mid-dorsal surface about midway between the ovary and the vitelline reservoir. After giving off Laurer's canal, the oviduct bears a small lateral diverticulum (the true receptaculum seminis) containing sperms. From this point onwards the oviduct runs almost parallel to the posterior border of the ovary as far as the opening of the common vitelline duct and then turns again towards the median line where it enlarges into a spacious muscular ootype, into which open numerous glands ("Mehlis gland"). The uterus arising from the distal end of the ootype immediately dilates to form a voluminous receptaculum seminis uterinum usually filled with sperms. Najarian (1954) described a similar part of the uterus in *Echinoparyphium flexum* serving "as depository for sperms." Beaver also referred to the presence of a "more or less voluminous receptaculum seminis uterinum" in *E. revolutum.* He also states that the absence of the seminal receptacle is typical of the genus *Echinostoma.* However, the receptaculum seminis as well as the receptaculum seminis uterinum are present in *E. nudicaudatum.* The receptaculum seminis uterinum narrows to become the uterus, which passes forward, ventral to the ovary, and is thrown into 10 to 20 coils in the region of the body between the ovary and the acetabulum. The number of the intrauterine eggs, in a sample of six permanent preparations, varied between 300 to 800. The uterus terminates as a muscular metraterm which typically traverses the right side of the cirrus sac to open through the female genital pore (fig. 9).

The vitelline follicles extend from about the level of the posterior border of the acetabulum (or only slightly posterior to it) almost to the posterior end of the body. The vitelline follicles at the level of the intestinal ceca are almost entirely extracecal with exception of one or two which may encroach into the intercecal space, while dorsally as well as ventrally to the intestine they may extend towards the median line, and are most numerous behind the posterior testis, but the two fields never become confluent. The transverse vitelline ducts and the median vitelline reservoir are dorsal to the recptaculum seminis uterinum and the ootype.

The female genital aperture is situated to the right of the male aperture; thus, both the male and the female apertures open in a common genital pore.

Excretory system: A careful search for flame cells was made in 13 living adult specimens but none were observed. Ciliary patches in the secondary lateral tubules were readily visible as distributed in the cercaria. Similarly, Johnson (1920) could observe no flame cells but reported finding "cilia in the main collecting tubes." It

is questionable whether these ciliated patches observed by Johnson were really present in the main tubules, since cilia were certainly not present in the main tubules of *E. nudicaudatum;* and such cilia are, in the cercariae, characteristically found in the secondary tubules. Tubangui (1932) reported the presence of 64 and 63 pairs of flame cells in *Euparyphium murinum* and *E. revolutum,* respectively, but did not mention the presence of cilia in the secondary tubules; however, his illustration of "flame cells" indicates that he was probably dealing with patches of cilia inside the secondary tubules rather than flame cells.

The primary, secondary, and main collecting tubules follow courses similar to those in the cercaria and eventually lead to a median tube that occupies the greater part of the post-testicular region along the median line. The excretory system on the whole is non-epithelial and thus agrees with LaRue's (1957) placing of the family Echinostomatidae in the superorder Anepitheliocystidia. The main collecting ducts and the unpaired terminal portion contain very fine "granulations," most densely distributed in the distal region of the bladder. These "granulations" are quite different in form from those present in the excretory system of the cercaria. The unpaired terminal part of the excretory system gives off lateral branches which are difficult to distinguish in whole mounts; in one specimen of *E. nudicaudatum* I have identified two branches on one side and one on the other side.

Relationships: Beaver (1937) critically compared *E. revolutum* with each of the 18 species of echinostomes which were known at that time to have 37 collar spines; he reduced 8 of them to synonymy with *E. revolutum;* the validity of the other 8 species was questioned and the 2 remaining were accepted as being distinct from *E. revolutum* and belonging to a different genus. He does not mention two new 37-collar spined species erected by Yamaguti (1934; 1935): *E. ralli* and *E. robustum.*

Recently Skrjabin (1956) and Yamaguti (1958) call attention to five new species of the genus *Echinostoma* characterized by 37 collar spines. Thus, in determining the specific identity of *E. nudicaudatum,* comparison must be made with all the 37-spined species of the genus: *E. revolutum, E. ralli, E. robustum, E. lindoensis* (Sandground and Bonne, 1940); and five species, namely, *E. revolutum tenuicollis* (Baschkirova, 1941), *E. goldi* (Oschmarin, 1956), *Echinostoma* sp. (Sadovskaja, 1952), *E. splendens* (Verma, 1936) and *E. stromi* (Baschkirova, 1946), which Skrjabin mentions in his monograph.

Echinostoma nudicaudatum bears a remarkable similarity to the other nine species. It differs from *E. revolutum, E. robustum, E. lindoensis, E. revolutum tenuicollis, E. stromi,* and *E. splendens* only in the absolute sizes of the collar spines, and from *E. ralli, E. goldi* and *Echinostoma* sp. only in the arrangement of the spines. In *E. ralli* there is a group of four corner spines and in *E. goldi* a group of six corner spines, whereas in *E. nudicaudatum* there is a group of five corner spines. In *Echinostoma* sp. the pattern of the cephalic spination is not known, but as is shown in the diagram (Skrjabin, 1956, fig. 84a) there are three angle spines on one side of the pharynx and only one on the other side, while the rest of the spines are arranged in an alternate fashion; this arrangement of the collar spines is in contrast with that found in *E. nudicaudatum.* It is now pertinent to consider the validity of this difference in absolute lengths of collar spines as a criterion distinguishing the species of 37-spined echinostomes. Yamaguti (1934), Bonne, Bras and Joe (1948) and Bras, Bonne and Joe (1953) have all considered the size of

spines to be of diagnostic value; and in the present investigation there has been found to be a significant difference between the spines of *E. nudicaudatum* and the other five species. Thus on the foregoing evidence there are grounds for proposing a new species of echinostome to include the parasites raised from larvae from Edgbaston Pool. Fortunately, however, a detailed knowledge of the larval stages of these parasites yields much stronger evidence in favor of the erection of a new species. The cercaria of *E. nudicaudatum* is clearly different from that of the cercaria of *E. revolutum* as described by Miller (1936), Beaver (1937), Johnston and Angel (1941), in the complete absence of a finfold in the tail and in the contents of the cystogenous gland cells which are "rod-like" in the cercaria of *E. revolutum* (Faust, 1918, p. 101; Miller, 1936, p. 43) while they are granular in the cercaria of *E. nudicaudatum*. A third distinguishing feature is the number and arrangement of flame cells; in the cercaria of *E. nudicaudatum*, there are at least 94 flame cells in all, but in the cercaria of *E. revolutum* according to Beaver, there are probably 36, but possibly more.

It is concluded, therefore, that a new species, *Echinostoma nudicaudatum* (Latin, *nudus*, naked; *cauda*, tail), must be proposed for the echinostome raised in pigeons from the cercaria found in *L. stagnalis* from Edgbaston Pool.

SUMMARY

The life cycle of *Echinostoma nudicaudatum* has been described with a detailed morphological study especially of miracidium, cercaria, and adult. There are at least four successive redial generations. The cercaria is without a finfold and the contents of the cystogenous glands are granular. The snail *Limnaea stagnalis* serves as the first intermediate host, whereas the second intermediate host may belong to the same species of *Limnaea* or to different species of *Limnaea, Planorbis, Paludina, Bithynia,* or even a tadpole. *Metacercariae* 5 days old to more than 14 months old are infective to pigeons. The egg-laying stage of *E. nudicaudatum*, in pigeons, is reached in about 14 days.

ACKNOWLEDGMENTS

The author expresses his appreciation to Dr. J. Llewellyn, Zoology Department, University of Birmingham, England, for the help given during the course of this work, and to Professor D. L. DeGiusti, Wayne State University, for help in preparing the manuscript.

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EXPLANATION OF PLATE I

(Figures drawn with the aid of camera lucida unless otherwise stated)

FIGURE 1. General anatomy of miracidium (outline of organs by camera lucida).

FIGURE 2. Arrangement of epidermal plates.

FIGURE 3. An immature redia, side view (flame cells with free hand drawing).

FIGURE 4. A mature redia containing developing cercariae and germ balls, side view.

FIGURE 5. General anatomy of cercaria (outline of organs by camera lucida).

FIGURE 6. General anatomy of adult.

FIGURE 7. Cephalic spination, ventral view.

FIGURE 8. Cephalic spination, dorsal view.

FIGURE 9. Reconstruction of cirrus sac ventral view.

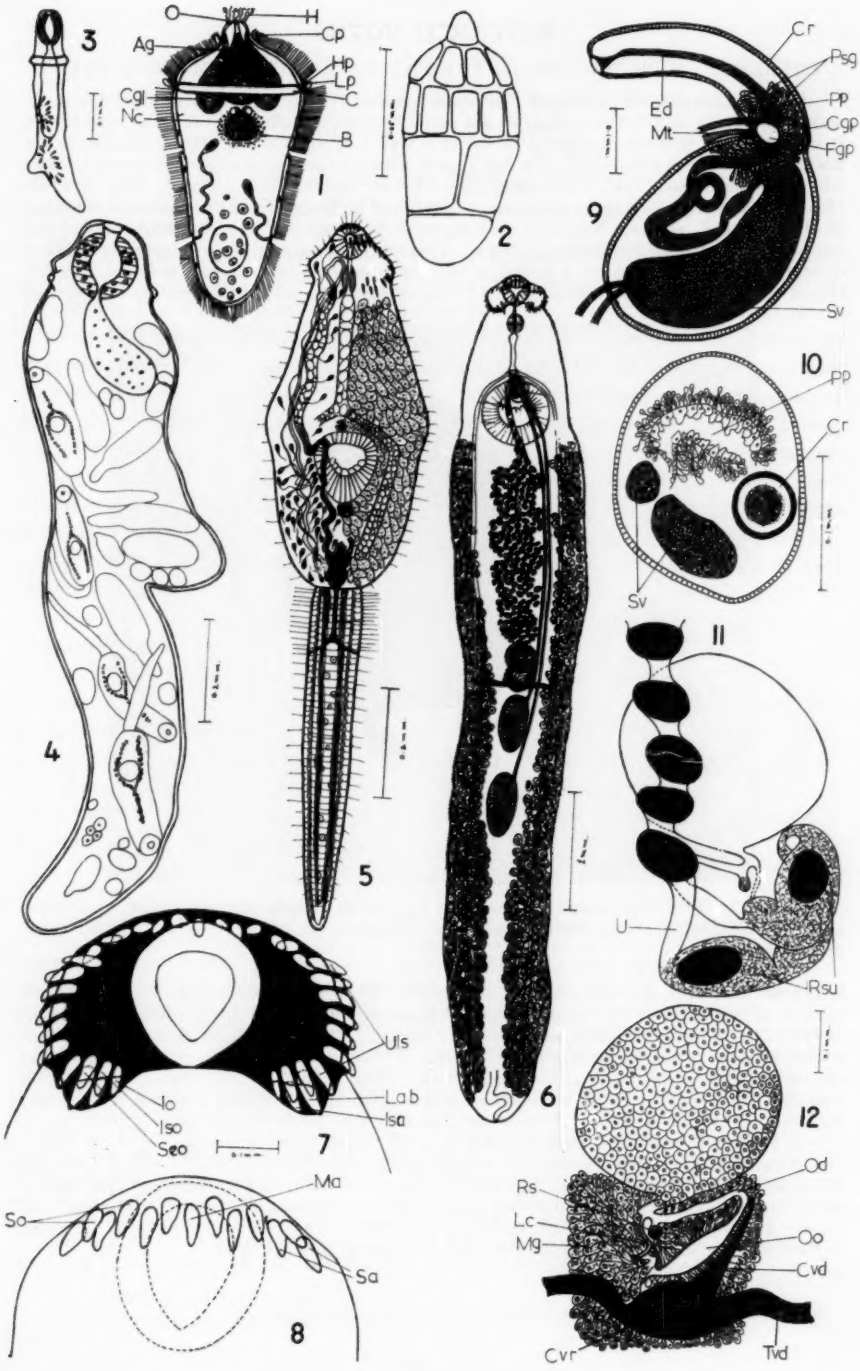
FIGURE 10. Frontal section of cirrus sac dorsal view.

FIGURE 11. Reconstruction of female genitalia, receptaculum seminis uterinum not shown, dorsal view.

FIGURE 12. Reconstruction of female genitalia, Mehlis' gland not shown, ventral view.

ABBREVIATIONS: Ag, apical gland; B, brain; C, collar; Cgl, cephalic gland; Cgp, common genital pore; Cp, conical protuberance; Cr, cirrus; Cvd, common vitelline duct; Cvr, common vitelline reservoir; Ed, ejaculatory duct; Fgp, female genital pore; H, hairs; Hp, hair-like process; Io, innermost oral corner spine; Isa, first aboral corner spine; Iso, first oral corner spine; Lab, lateral aboral corner spine; Lc, Laurer's canal; Lp, lateral process; Ma, median aboral spine; Mg, Mehlis' gland; Mt, metraterm; Nc, nuclei of nerve cells; O, opening of apical gland; Od, oviduct; Oo, ootype; Pp, pars prostatica; Psg, prostate glands; Rs, receptaculum seminis; Rsu, receptaculum seminis uterinum; Sa, spines of aboral series; Seo, second oral corner spine; So, spines of oral series; Sv, seminal vesicle; Tvd, transverse vitelline duct; U, uterus; Uls, unalternating lateral spines.

PLATE I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RESEARCH NOTES

PRESENCE OF PHENOLASE IN THE FASCIOLID METACERCARIAL CYST.

During studies on the biology of *Fascioloides magna* (Bassi, 1875) Ward, 1917, it was observed that newly encysted metacercariae are perfectly white in color, but gradually turn brown. It was suspected that this darkening of the cyst wall may represent a process similar to the tanning of the fasciolid egg-shell. The egg-shell is known to consist of a quinone-tanned protein, sclerotin (Stephenson, 1947, Parasitology **38:** 128–139; Smyth, 1954, Quar. J. Micr. Sci. **95:** 139–152). The catechol test for phenolase, as described by Smyth (ibid.), was therefore applied to cercariae of *F. magna.* Naturally shed cercariae were pipetted into 70 percent alcohol and washed by repeated sedimentations in water. They were then incubated in 0.2 percent catechol, at approximately 34 C. After a period of about 1 hour each cercaria had acquired a darkly tanned coat over most of its body (fig. 1). The anterior tip, bearing the oral sucker, did not

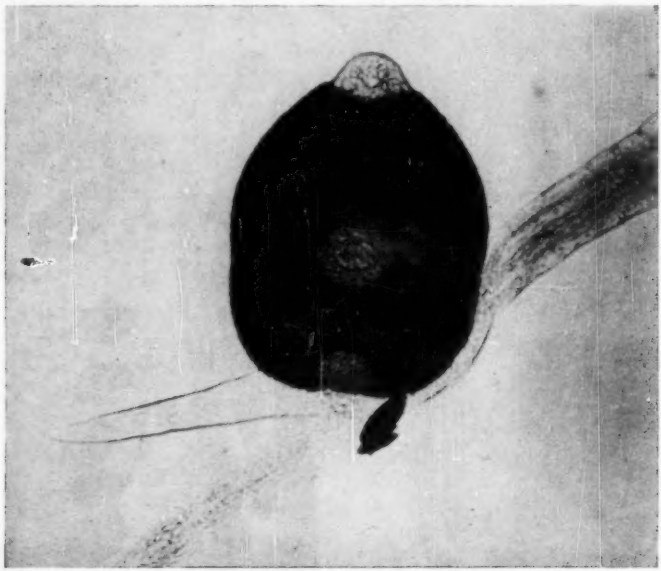

FIGURE 1. Photomicrograph of cercaria of *Fascioloides magna* after incubation in catechol, showing coating of tanned cystogenous material. The picture is traversed by the tail of another cercaria.

have a tanned coating dorsally, ventrally or laterally. The tanned material was also absent from the ventral surface in the region of the ventral sucker, and was absent at the point of attachment of the tail. No tanned material was present in the tail. The tanned layer consisted of densely packed granules. The irregularly placed bundles of short rods which are generally distributed near the surface of the cercaria, were not tanned. These findings indicate that the cystogenous granules of the fasciolid cercaria contain an enzyme capable of oxidizing a phenol, and support the belief that the natural color of the metacercarial cyst is due to quinone tanning.

A. F. Bird, in a personal communication to J. D. Smyth (cited by permission of Dr. Bird), also suggested an analogy between the cystogenous granules of a fasciolid cercaria (*Fasciola hepatica*) and the shell globules in the vitelline cells of the adult. Bird pointed out that tanning of the cyst did not occur in oxygen-free water, and cited chemical evidence that phenolic substances are present in the brown cyst.—W. C. CAMPBELL, *University of Wisconsin, Madison.* Present address: *Merck Institute for Therapeutic Research, Rahway, N. J.*

IMMUNOLOGICAL TOLERANCE STUDIES WITH MICE AND *TRICHINELLA**

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The concept of immunological tolerance was suggested by Burnet and Fenner (1949) on theoretical grounds. Actively acquired tolerance has been described by Billingham, Brent and Medawar (1953) as an induced, specific, central failure of the mechanism of immunological response brought about by an exposure of animals to antigenic stimuli before maturation of the faculty of immunological response. These workers and others have provided evidence for this hypothesis by experiments in which skin grafts were accepted by mice which had been previously injected during early life with tissue suspensions of the donor mouse. In other words, an immunological tolerance had been induced causing the mouse to regard this foreign substance as compatible tissue. Experiments on immunological tolerance have been performed in a variety of animals utilizing antigens such as foreign erythrocytes, skin grafts, tumors, chemical allergens, various purified proteins and, to a limited extent, with microbial agents (Weiss, 1958; Egdahl, 1958). Some evidence of acquired tolerance has accumulated to indicate its natural occurrence in vertebrates (Anderson et al, 1951).

To our knowledge the concept of actively acquired immunological tolerance has not been applied experimentally to helminth-vertebrate relationships, and with the possible exception of Kerr and Robertson's work with *Trichomonas* in cattle (1954), this concept has not had experimental application to problems within the field of parasitology. An experimental alteration of the relationship between a vertebrate and a parasitic nematode was attempted using the host-parasite combination of the white mouse and *Trichinella spiralis*.

MATERIALS AND METHODS

White mice (Fairfield-Webster strain) were bred in the laboratory to insure availability of newborn animals. The stock infection of *Trichinella spiralis* was maintained in Holtzman rats. Larvae for maintenance of stock infections, infection of experimental mice, and production of antigenic materials were recovered from rats after a 6- to 12-week period of infection (table I).

Antigenic materials consisted of two types: larval excretions and secretions hereafter called ES antigen, and sonicated larvae (table I). The production of ES antigen followed the method of Campbell (1955). ES antigens were discarded if less than 50 percent of the larvae were viable at the end of the 5-day incubation period. Loss of motility and internal disintegration of structure were criteria for larval death. Sonication resulted in disruption of larval bodies to the extent that intracardiac injections were possible without filtration or centrifugation.

Recovery of adult worms followed the procedures of Larsh and Kent (1949). The small intestine was slit longitudinally, cut into short lengths, and placed in a 0.05 percent NaOH solution. After refrigeration overnight, the intestinal fragments were removed and examined for worms, the remaining solution was centrifuged, and the sediment placed in petri dishes. Direct counts of adult worms were made under a dissection microscope.

The total larval worm burden of each mouse was obtained by the digestion procedure for rat muscle (table I), except that only the skin, paws, tail, and visceral organs were discarded.

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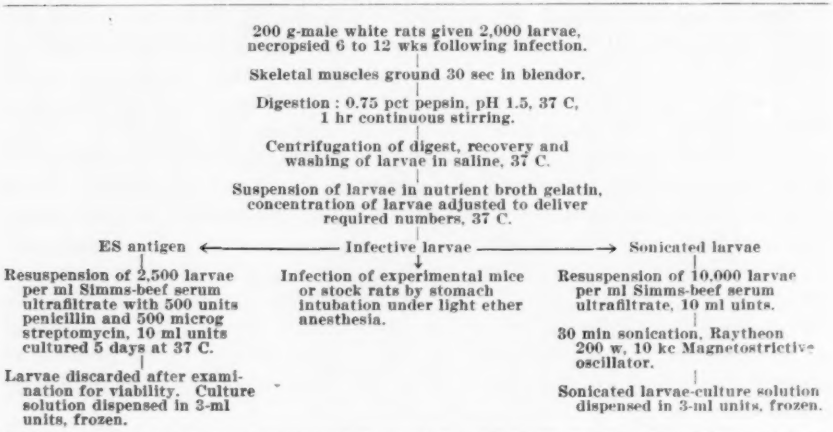
After centrifugation of the digest the larvae were recovered and suspended in 20 ml of a nutrient broth-gelatin solution. The numbers of larvae present in three 0.20-ml samples from this suspension were averaged with extrapolation to obtain the total number of larvae per carcass.

Intracardiac injections of newborn mice followed the method of Grazer (1958). A 30-gauge needle was attached to several inches of a small bore plastic tubing coupled to a 1-ml tuberculin syringe. Newborn mice were immobilized in a stretched position which makes the heart easily visible; the injection of 0.05 ml antigen into the heart was accomplished without serious difficulty or loss of animals. Approximately one-half of the newborns in a litter served as controls and

TABLE I. *Preparation of* Trichinella *antigens.*


200 g-male white rats given 2,000 larvae, necropsied 6 to 12 wks following infection.
Skeletal muscles ground 30 sec in blendor.
Digestion: 0.75 pct pepsin, pH 1.5, 37 C, 1 hr continuous stirring.
Centrifugation of digest, recovery and washing of larvae in saline, 37 C.
Suspension of larvae in nutrient broth gelatin, concentration of larvae adjusted to deliver required numbers, 37 C.
ES antigen
Infective larvae
Sonicated larvae
Resuspension of 2,500 larvae per ml Simms-beef serum ultrafiltrate with 500 units penicillin and 500 microg streptomycin, 10 ml units cultured 5 days at 37 C.
Larvae discarded after examination for viability. Culture solution dispensed in 3-ml units, frozen.
Infection of experimental mice or stock rats by stomach intubation under light ether anesthesia.
Resuspension of 10,000 larvae per ml Simms-beef serum ultrafiltrate, 10 ml uints.
30 min sonication, Raytheon 200 w, 10 kc Magnetostrictive oscillator.
Sonicated larvae-culture solution dispensed in 3-ml units, frozen.

were similarly injected with Simms-beef serum ultrafiltrate-antibiotics. Experimental and control mice were marked when injected and the litter returned to the mother until weaned.

The criterion for induced tolerance in these studies was a statistically significant increase in the worm burden of experimental as compared to control mice. Statistical analysis was based on the Student "*t*" test (Snedecor, 1956) with no significance given to differences below the 5 percent level.

RESULTS

Newborn mice were given 0.05 ml antigenic material by intracardiac injection. Six weeks later a challenge of 200 *Trichinella* larvae was administered. Adult and larval recoveries were made at 13 and 30 days, respectively, after challenge. Control animals were similarly treated except that Simms-beef serum ultrafiltrate was injected. The results of these experiments are shown in table II.

In experiment 1 a mean recovery of 21 adult worms was obtained in experimental animals injected with ES antigen at birth as compared to a mean of 24 in controls. Larval recoveries from experimentals averaged 41,500 as compared to a mean of 47,500 in controls. In experiment 2, sonicated material was used as the antigen. The procedure was identical to experiment 1 except that only larval recoveries were made. Twenty animals per group were used with a mean recovery of 53,500 in experimentals as compared with 55,500 in controls. Experiment 3 represents animals injected with sonicated material as above, but with an additional 0.05 ml given each animal 5 hrs after the first injection. Sixteen experimentals and 15 controls were sacrificed for adult worm recoveries with a mean of 11 and 12 worms, respectively. These means are lower than expected, although uniformly low for both experimental and control animals; viability of infective larvae did not appear to be a significant factor since control mice infected with 200 organisms from the same pool

TABLE II. *Effect of intracardiac injections of newborn mice with* Trichinella *antigens on subsequent worm burdens from a single infection of 200 larvae.*

Exp. no.	Group: injection	Adult worm recoveries			Larval worm recoveries		
		No. mice	Mean	(S.E.)	No. mice	Larval mean	(S.E.)
1	A: ES*	15	21	(8)	15	41,500	(4,500)
	B: control*	20	24	(7)	19	47,500	(8,500)
2	A: sonicate*				20	53,500	(4,000)
	B: control*				20	55,000	(4,000)
3	A: sonicate**	16	11	(6)	26	33,000	(4,000)
	B: control**	15	12	(6)	21	35,500	(5,500)

* Indicates single injection of 0.05 ml.
** Indicates two injections of 0.05 ml with a 5-hr interval.

of infective larvae harbored an average of 69 worms at necropsy 7 days after infection. The remaining animals were sacrificed 30 days after challenge for larval recoveries with means obtained of 33,000 and 35,500 for experimental and control animals, respectively.

An attempt was made to determine the effect of intracardiac injection of ES antigens in newborn mice on their ability to acquire resistance through sensitization with living larvae to a challenge infection (table III). All mice were similarly injected at birth with 0.05 ml of ES antigen or control solution. When 6 weeks of age the mice were divided into 6 groups: mice in groups A and B received two sensitizing infections at 6 and 9 weeks of age, and a challenge infection at 13 weeks of age; mice in groups C and D received only the challenge infection; mice in groups E and F received only the two sensitizing infections. All mice were necropsied 13 days after the time of the challenge infection. An analysis of the differences between adult worm means in groups A and C (ES injected) as compared to their respective control means in groups B and D showed these differences to be statistically insignificant. Worm recoveries from mice in groups C and D, and E and F

TABLE III. *Effect of intracardiac injections of newborn mice with* Trichinella *antigens on subsequent worm burdens from repeated infections with larvae.*

Exp. no.	Group: injection	No. larvae/mouse given at ages below:			Worm recoveries			
		6 wks	9 wks	13 wks	No. mice	Adult mean	(S.E.)	Larval mean*
4	A: ES	200	200	400	41	20	(6)	38,500
	B: control	200	200	400	36	10	(6)	53,500
	C: ES			400	15	55	(17)	< 750
	D: control			400	12	40	(14)	< 750
	E: ES	200	200		10	1		41,000
	F: control	200	200		10	0		41,500

* Standard errors unavailable due to pooling of mice during digestion.

demonstrated that larval recoveries in groups A and B essentially represented a summation of progeny produced by adults from the first two infections and that the adult worms recovered from groups A and B were present as a result of the challenge with 400 larvae. The significant differences between adult worm means in groups A and B as compared to groups C and D is taken as evidence of the increased resistance to *Trichinella* developed in mice as a result of previous infection and sensitization with living larvae.

The immunogenic properties of ES and sonicate antigens were also evaluated in adult mice. Experimental mice (5 to 6 weeks of age) were each given by the ip route a total of 0.9 ml of ES or sonicate antigens in 6 divided doses at 3 day intervals. Control mice were similarly treated except for material injected. Two weeks after the last ip injection, each mouse was given 200 viable larvae. Necropsies were

TABLE IV. *Effect of intraperitoneal injections of adult mice with* Trichinella *antigens on subsequent worm burdens from a single infection of 200 larvae.*

Exp. no.	Group: injection	Adult worm recoveries			Larval worm recoveries		
		No. mice	Mean	(S.E.)	No. mice	Mean	(S.E.)
5	A: ES	26	21*	(5)	27	20,000*	(2,000)
	B: control	27	49	(5)	26	39,000	(2,500)
6	A: sonicate	11	25	(7)	12	28,000	(2,500)
	B: control	11	18	(6)	12	28,000	(1,500)

* Indicates difference between groups significant at 1 pct level.

done 13 or 30 days after infection for adult or larval recoveries, respectively (table IV). Mice injected with ES antigen were found to harbor significantly fewer worms at necropsy than control mice (adult means: 21 and 49, respectively; larval means: 20,000 and 39,000, respectively). On the other hand, mice similarly injected with sonicate antigen yielded mean recoveries of worms which were not significantly different from those in control mice (adult means: 25 and 18, respectively; larval means: 28,000 and 28,000, respectively).

DISCUSSION

The criterion in these studies for acquired tolerance was the demonstration of a significantly greater worm burden in experimental as compared to control animals at a specified time after uniform challenge. By this criterion acquired tolerance was not demonstrated in these experiments. Due to possible variables in an experimental design for such studies, these results must be interpreted as tentative and preliminary, and do not rule out the possibility of inducing tolerance in the mouse to *Trichinella*. One such variable is the timing of injections and challenge infections. The schedule in these experiments, i.e., injection at birth with challenge 6 weeks later, was arbitrarily chosen in the absence of precedent for this system on the basis of similar schedules employed for other systems. The optimal quantity and quality of antigens injected and timing of injections or challenge infections with respect to the strain characteristics of the host are problems which have not been fully resolved for this system. In this connection we are currently doing transabdominal intrafetal injections of mice with ES antigen in an attempt to find a more favorable time of injection; these experiments have not progressed to allow comment on the results obtained. We have discarded the use of sonicate antigen after finding no marked change in the worm burdens of mice which were injected with this material at birth or as adults and subsequently challenged with larvae. Our results with ES antigen in adult mice support the findings of Campbell (1955) that this antigen has immunogenic properties when given by the ip route.

In related studies (Olson and Ewert, unpublished), we have assayed the antigenic content of the ES and sonicate materials used for injection in these experi-

ments by a modification of the micro agar gel technique of Wadsworth (1957). When the above antigens are tested against antisera from rabbits immunized by living larvae, a number of precipitation zones are detected which indicate the complexity of ES antigens and, in particular, the sonicate antigens. While some zones from the above two materials show coalescence against this antiserum, differences also exist which may be related to the difference in immunogenicity of these two antigens noted in the experiments above (table IV).

No serological studies have been done on mice in these experiments; only the worm burdens at necropsy have been used as criteria for tolerance. Serology was not scheduled in the design of these experiments unless data on worm burdens indicated a state of tolerance had been induced. Possibly the lack of concomitant serology on these animals is a criticism of our conclusions, i.e., the injection of *Trichinella* antigens into the newborn may have altered the serological response to infection without an accompanying tolerance in worm burdens. However, the significance of circulating antibody, local tissue responses, and the possible role of hypersensitivity reactions do not seem at present adequately understood in terms of the resistance of the sensitized mouse to *Trichinella spiralis* to preclude the value of worm burdens as critical evidence of host tolerance to this parasite.

It is of interest to speculate on the possible role played by actively acquired immunological tolerance in the evolution of present and new host-parasite relationships. It is known that a variety of parasites will invade the fetal host (e.g., *Toxocara canis, Toxoplasma gondii*); conceivably, acquired immunological tolerance may be a factor in altering the host-parasite relationships resulting from such infections. Furthermore, one can speculate on the probability of similar exposure between fetal or newborn vertebrates and antigens from free-living invertebrates living in a common ecology with repeated contact.

SUMMARY

Attempts to induce acquired immunological tolerance in white mice to *Trichinella spiralis* have been negative in these experiments. Intracardiac injections of newborn mice with ES antigens (excretions and secretions of incubated larvae) or sonicated larval material have not resulted in a significant increase (or decrease) in the mean adult or larval worm burdens in mice necropsied after single or repeated infections with *Trichinella* larvae as compared to similar means in suitable control infections.

The intraperitoneal injections of ES antigen were found to increase significantly the resistance of adult mice to a challenging infection while similar injections of sonicate antigen did not provoke this protection against challenging larvae. The ES antigen appears to have immunogenic properties not found in the sonicate material.

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RESEARCH NOTE

EXPERIMENTAL INFECTION OF NEONATAL MICE BORN OF MOTHERS INFECTED WITH *SCHISTOSOMA MANSONI*.

The question of whether congenital transfer of resistance occurs in schistosome infections has apparently not been investigated. The present report describes three experiments in which offspring of females that had received a series of immunizing infections with *Schistosoma mansoni* were infected with the homologous parasite.

CFW female mice were exposed five or six times to 10 to 12 cercariae of *S. mansoni* obtained from pools of at least eight infected *Australorbis glabratus*. The interval between exposures was 2 to 3 weeks. Infected and non-infected females were mated the day following the last immunizing exposure and litters were born 4 to 7 weeks later. Offspring were infected when 1 to 6 days of age by placing each mouse in a vial containing 10 or 25 cercariae in a small volume of spring water. After an exposure period of about 30 minutes, the young mice were returned to their own mothers for nursing. In each experiment, the ages of the test and control litters were comparable and all exposures to cercariae were carried out at about the same time with cercariae from the same pool. The litters were sacrificed 5 to 8 weeks after infection and schistosomes recovered by perfusion.

Results are summarized in table I. In the first experiment, the infected mothers harbored

TABLE I. Schistosoma mansoni *worm burdens after neonatal infection of mice born of normal and infected mothers.*

Experiment	Number of mice (Number litters)		Number of cercariae per mouse	Worm burden	
				Mean	S.D.
1. Test	21	(3)	10	2.8	1.5
Control	15	(3)	10	3.9	1.7
2. Test	34	(6)	10	5.6	1.3
Control	34	(7)	10	5.6	1.9
3. Test	23	(4)	25	12.3	3.5
Control	23	(4)	25	13.9	3.5

an average of 4.3 worms and in the next two experiments, the mothers had an average worm burden of 13.1 worms. All infected mothers showed numerous liver lesions at autopsy. The differences in mean worm burden between test mice born of infected females and control groups born of normal females were not statistically significant ($P > 0.1$). No detectable differences in worm maturation between test and control groups were noted. Since Moore et al (1949, J. Parasit. **35:** 434) failed to infect mice with *S. mansoni* in utero, it is highly probable that all parasites recovered from offspring were derived from the challenge infection. Although it was not determined in fact that the infected mothers had developed an acquired immunity there is good evidence that repeated exposure to small numbers of cercariae increases the resistance of mice to a subsequent challenge infection (Kagan, 1958, Rice Inst. Pamph. **45:** 151–183).

These experiments indicate that the young born of mothers who had been exposed to cercariae of *S. mansoni* 5 to 6 times at 2 to 3 week intervals were as susceptible to *S. mansoni* infection as the neonatal mice from the uninfected control mothers.

(This investigation was supported by Research Grant E-1893 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, U. S. Public Health Service.)—CLARENCE J. WEINMANN, *Department of Microbiology, College of Medicine, University of Florida, Gainesville.*

THE EFFECT OF BODY TEMPERATURE ON THE DEVELOPMENT OF *TRICHINELLA SPIRALIS* IN BATS

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Any attempt to determine the in vivo temperature tolerance of a parasite would be limited by the temperature tolerance of the host. Most mammalian species have a narrow body temperature range. Hibernating mammals provide a striking exception and undergo drastic seasonal variations in body temperature. The temperature variations tolerated by bats are greater than those of other hibernators, since their body temperature drops to within a few degrees of ambient temperature whenever they are inactive. (Hock, 1951; Lyman and Chatfield, 1955)

In the experiments reported here, an attempt was made to define the temperature tolerance of *Trichinella spiralis* by following the course of experimental infections in bats held at different temperatures. Preliminary experiments (Chute and Covalt, 1958) indicated the parasite would develop to the adult stage in the intestines of bats held at 34 C, but not in bats at 23 C or 5 C.

MATERIALS AND METHODS

The bats were collected from two sources, both in central western Vermont. Hibernating males were taken from caves in mid-winter. It can be assumed that these bats were at least 8 months old and had been hibernating for at least 3 months. The majority of bats taken in hibernation were *Myotis lucifugus;* a few were *Pipistrellus subflavus* and *Myotis keenii.*

Bats used in experiments 3 through 5 were collected in a summer "nursery" roost in a hotel attic. Only *Myotis lucifugus* were captured in the summer roost. The animals used in experiments 3 and 4 were collected in mid-June and were all pregnant or lactating females. Those used in experiment 5 were collected several weeks later, and many were bats-of-the-year of both sexes while the remainder were mature females.

The strain of *Trichinella spiralis* used to infect bats was originally obtained from stock maintained by the Communicable Disease Center of the U.S. Public Health Service and had been maintained in this laboratory in white rats. The methods used in isolating, counting, and administering the larvae have been described previously (Chute, 1956). Larvae used in each experiment were isolated from different stock animals.

Bats held at 26, 30 and 34 C, were kept individually in 1-pint cylindrical ice cream cartons prepared in a manner similar to that described by Constantine (1952). The bats held at 5 and 23 C were kept in larger groups in 2-cu-ft screen cages. Before infection, each bat was trained to eat living *Tenebrio* larvae from forceps. Bats at the higher temperatures were hand fed 10 to 30 *Tenebrio* per day. The bats used in experiments 3 through 5 received a liquid vitamin-mineral supplement (Rexall Super Plenamins Jr.) as suggested by Orr (1958).

Temperature for the experiments at 34, 30, and 5 C was controlled within 1 degree C in a refrigerated cold room and in electric incubators. The 23 and 26 C temperatures represent laboratory temperatures, and were controlled to plus or minus 2 C. In experiments 1 and 2, the bats held at 5 C were aroused for feeding every 3d day. In subsequent experiments, bats held at 5 C remained at that temperature throughout the experiment.

The entire intestine of infected bats was examined by inverting sections of intestine on toothpicks and incubating the sections in acidified saline at 45 C (McGee, Russomanno, and Sandground, 1957). Worms were counted in the petri dishes in which the sections were incu-

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bated, then transferred to depression slides for microscopic determination of sex and stage of development. Some worms from each host (at least 10 where possible) were fixed in hot FAA and mounted in lacto-phenol for more exacting examination.

RESULTS

Experiment 1. Male bats collected during hibernation were inoculated with 500 trichina larvae each. Ten bats were maintained at 5 and 10 at 34 C. On the 2d day of infection and at intervals thereafter, one bat from each group was killed and examined. Larvae were recovered from the intestines of all of 7 bats in the 5 C group examined on the 6th, 7th, and 8th days. Of special interest are the two adult worms found in bats held at 5 C. Both these adults, recovered from two *myotis lucifigus* on the 2d and 6th days after infection, were females of normal size and morphology.

In the intestines of all ten bats held at 34 C adult worms were found. The average recovery was 25. Twenty-six larvae were also found in the bat killed on the 2d day after infection.

Experiment 2. Two groups of 6 bats each were infected with 100 trichina larvae each and the groups kept at 5 and 23 C. All bats were males captured during hibernation. Starting on the 2d day of the infection, one or two bats were killed each day. No adult worms were found in either group. Larvae were found in four of the six bats held at 5 C, with an average recovery of 10. An average of 20 larvae were recovered from 5 of 6 bats at 23 C.

Experiment 3. Female bats captured in a summer roost were divided into three groups. Eight animals were maintained at 5, seven at 30 C, and 12 at 34 C. All were infected with 500 larvae. On the 3d day of the infection and at intervals thereafter, animals from each group were killed and their intestines examined. Two bats in the 34 C group were examined by pepsin-HCl digestion on the 30th day of infection.

Larvae only were recovered from the 5 C group, all of which were infected. The average recovery was 18. In the 30 C group, larvae were found in the two animals examined on the 3d and 4th day. The recoveries from five bats of this group examined from the 5th through the 9th day averaged 38 adults per host. In the 34 C group larvae were recovered during the first 6 days from three of four hosts examined, the fourth, killed on the 5th day, yielding 3 adult worms. Six animals of this latter group killed from the 7th through the 12th day all contained adult worms, an average of 29 per host.

Two apparently normal and live muscle larvae were recovered from one of the two bats maintained at 34 C for 30 days.

Experiment 4. Twenty-seven bats captured in summer roosts were divided into a group of 10, maintained at 26 C, and a group of 17 at 30 C. All animals were given an initial infection of 100 larvae and an additional 30 larvae were administered on the 5th day after infection.

Recoveries from the 30 C group were very low. One larva was found in a bat killed on the 3d day. Adults were recovered from 9 of 10 bats killed between the 8th and 22d day of infection. Recoveries averaged nine per infected bat. No worms were recovered from six bats of this group killed between the 4th and 8th day of infection. It is possible that the second infection with 30 larvae accounted for most of the worms recovered in this group, since only one worm was recovered from the

first 7 animals examined.

No trichina were recovered from any of the 26 C-group. The negative results must be considered together with the very low recoveries in the 30 C-group of this experiment and the development of intestinal infections with larvae in the 23 C-group in Experiment 2 above. It seems probable that the trichina larvae administered had a lower vitality than those used in earlier experiments.

Experiment 5. Eighteen bats taken from summer roosts were infected with 575 trichina larvae each. All bats were kept 26 C and were examined between the 3d and 15th day of infection. Only four of the 18 were found to harbor intestinal trichina. All the worms were larvae. The total recovery was seven worms.

DISCUSSION

The results presented here indicate an in vivo temperature tolerance for *T. spiralis* with a lower limit between 26 and 30 C. The present results refer only to the development of intestinal stages of the parasite and are open to the objection that the bat is probably not to be considered as a normal host. A more acceptable definition of the temperature tolerance of *T. spiralis* requires work with a host combining normal susceptibility with the capacity to undergo the requisite changes in body temperature for several days at a time. The upper limit is not defined by these experiments but the reports of infections in birds, such as that of Augustine (1933), indicate an upper limit higher than normal mammalian temperatures.

The very limited development of muscle larvae in the two bats held for 30 days at 34 C suggests an abnormal host relationship and probably precludes the possibility of natural infections in bats. The insectivorous diet of most bats also makes natural infection unlikely. It should be noted, however, that the report of Merkushev (1955) concerning the experimental use of beetles as transfer host for *T. spiralis* makes natural infection a little more probable.

The development of a small number of adult worms in bats at 5 C is probably explained by the fact that the bats in question were aroused every few days for feeding and that they were kept, as a group, in a large cage as opposed to the individual cages used in other experiments. Under these conditions, it is possible that the body temperature of the bats approached the normal mammalian level for considerable periods of time. In later experiments, in which the 5 C-group was not aroused for feeding, no adults were found. The development of some adults at 5 C suggests that a period of uninterrupted exposure to moderate temperature, as in the 23 C- and 26 C-groups, might be more effective in retarding development of the parasite than briefer, interrupted exposure to much lower temperatures. This idea is in accord with the results of Simitch and Petrovitch (1954) who determined the length of uninterrupted hibernation required to eliminate a number of species of helminth parasites from the European ground-squirrel.

As evidenced in these experiments, the effect of lowered host temperature is inhibitory rather than lethal, since the majority of the recovered worms, even after the longest exposures at below normal temperatures, were alive and appeared normal. This inhibitory or retarding effect of low temperature is in accord with the observations of Rodhain (1951), who used *Toxoplasma* in marmots, and of a number of earlier authors, who used bacterial and protozoan parasites in a variety of hibernators (Jadin, 1932).

SUMMARY

1. *T. spiralis* will regularly develop to the adult stage in the intestine of bats held at 34 and 30 C, but only two adults were found in 58 infected bats held at 5, 23, and 26 C.

2. Two muscle larvae developed in one of two bats infected with 500 larvae and held at 34 C for 30 days.

3. Lowered host temperature, for the periods of time reported, may inhibit the development of the parasite without resulting in their elimination from the host.

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STUDIES ON BOVINE GASTRO-INTESTINAL PARASITES. XXI. IMMUNITY TO *COOPERIA PUNCTATA* AND *OESOPHAGOSTOMUM RADIATUM*

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Much interest is being shown at present in the subject of immunity, and the phenomenon is being demonstrated in infections with many species of parasites. The following papers will be found to contain many references of interest to the reader: Mayhew (1940) (1941), *Haemonchus contortus,* hookworm and nodular worm in calves; Vegors (1954), *Strongyloides papillosus* in calves; Stoll (1958), *Haemonchus contortus* in sheep; Jarrett et al (1959) vaccination against *Dictyocaulus viviparus* in calves.

CARE OF ANIMALS AND EXPERIMENTAL PROCEDURES

The calves were obtained as soon after birth as possible, usually within 1 or 2 days, and kept in individual cages in the animal building of the Veterinary Science Department. These cages have floors raised above the cement floor of the room to facilitate cleaning. The cages and floor of the room were cleaned three times a week, washed first with cold and then with steaming hot water. The hay came from ground that had not been pastured for several years and the water from the Baton Rouge city water system. The same brand of commercial grain concentrate was fed throughout the duration of the project.

The animals were inoculated with larvae of pure infections of known species as indicated. The cultures were made by placing the manure in wire baskets 12 inches square and 2 inches deep, and incubated at 70 F for 10 to 14 days. The larvae were isolated from the manure by placing the culture baskets in 14-inch-square Baerman funnels overnight. The larvae were allowed to settle to the bottom in 50-cc centrifuge tubes for 24 hours. Estimates of the number of larvae were made by placing two drops of 15 to 20 cc of the suspension of the larvae on a microscopic slide for examination under the microscope. Estimates of the total number present per cc were then determined. The desired number of larvae was then placed in the grain ration and fed to the calf. Numbers of larvae administered, however is a very unreliable way to predict the resulting egg counts or severity of symptoms because of the unknown host-parasite relationships. Even equal extremely large numbers of larvae of the same culture may cause the death of one animal and produce no symptom in another of approximately the same age and breed.

The egg counts were made from manure removed from the rectum during the early forenoon, placed in 50-cc centrifuge tubes, and allowed to stand in water till afternoon. After centrifugation in an International clinical centrifuge, the water is replaced by a solution of cane sugar with a specific gravity of 1.16 and again centrifuged. The eggs are removed with a wire loop for counting. The tubes are weighed, the total sediment computed, and the egg counts per gram determined. Four tubes are examined from each sample which contain usually from 60 to 80 g of sediment. By the consistent use of this procedure the range of variation in counts and the detection of very small numbers of eggs when they are first due to appear after inoculation and when they are disappearing with the development of immunity can be very accurately determined.

RESULTS

No. 281B. This animal was a purebred Holstein male born October 9, 1956. He was inoculated January 30, 1957, with 146,850 *Cooperia punctata* larvae; the first eggs were recovered February 11 (fig. 1). High counts were maintained until February 26, followed by a drop to between 0 and .01 EPG (eggs program) in four counts between March 6 and 9. A range 3.9 to 26 EPG was then maintained

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between March 11 and June 12. To shorten the graph only the range and number of counts between April 15 and May 24 are shown. A sudden drop followed, and three negative examinations were recorded June 22, 24, and 25. A period of increase occurred to a maximum of 0.74 EPG between July 1 and August 1. The calf was reinoculated July 27 with 162,160 and August 19 with 18,750 *C. punctata* larvae (fig. 1). Following August 12, less than 0.18 EPG were recovered in 30 examinations up to November 4.

The sudden drops in egg counts followed by increases usually to a lower range, we believe, are unsuccessful attempts to establish complete immunity. Many instances of this will be found to occur in the descriptions of the other calves in the following pages.

Nodular worm larvae (18,750) were also included in the inoculation of August 19 and the first eggs of this species were recovered October 3, 45 days later (fig. 1). The resulting nodular worm egg counts varied between 10 and 34 EPG until October 19. This shows that immunity to *C. punctata* does not protect against nodular worm infection.

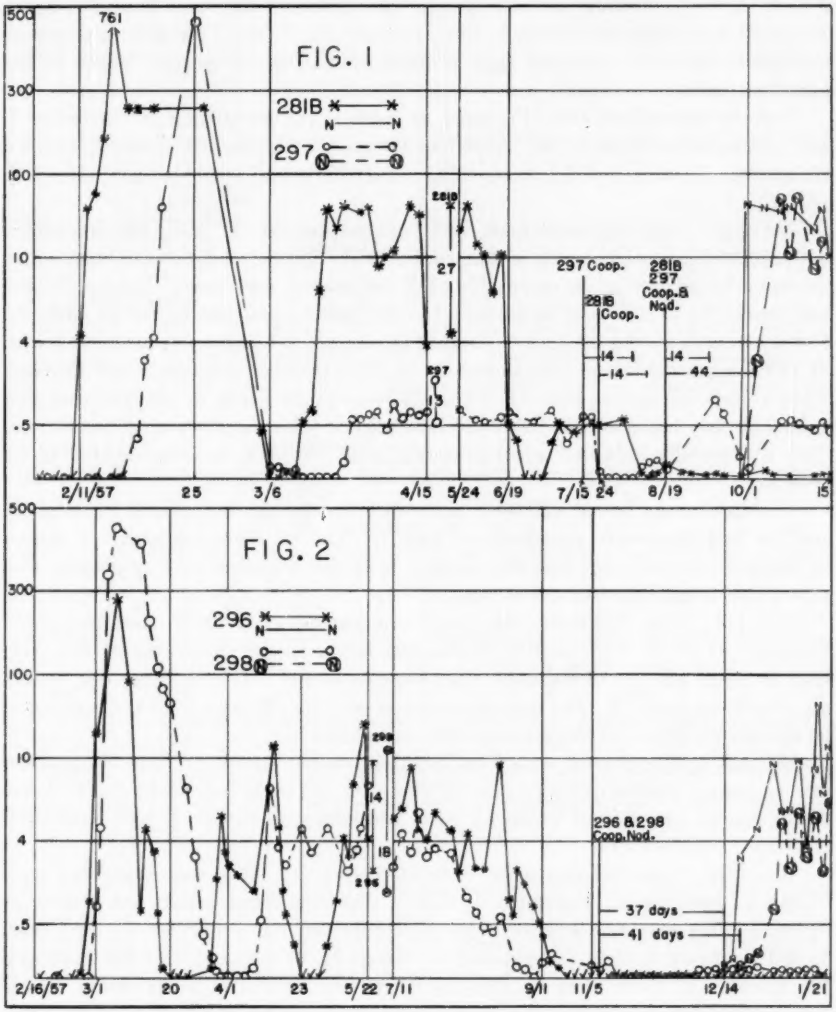
No. 297. This animal was a purebred Jersey male born at the L.S.U. Dairy on October 19, 1956, and inoculated February 5, 1957 with 150,000 *C. punctata* larvae. The first eggs were recovered (fig. 1) on February 18 and the numbers increased to a maximum of 466 EPG on February 25. The numbers then declined very rapidly, and no eggs were recovered in five examinations between March 11 and 18. Eggs were recovered again beginning March 20, and a low but uniform range between 0.6 and 2.2 EPG was maintained until July 22. Thirteen examinations between April 15 and May 24 (details not shown) varied between 0.67 and 2.18 EPG. Five examinations between July 25 and 30 were negative. Thirty-five counts between July 31 and November 4 had a maximum of 0.66 EPG. This series of low egg counts occurred in spite of reinoculation with 25,960 *C. punctata* larvae on July 15 and 18,750 *C. punctata* on August 19, 1957.

Nodular worm larvae (18,750) were also given August 19, 1957 (fig. 1), and the first eggs were recovered October 2, or 44 days later. This is another demonstration that immunity to *C. punctata* is not effective against the nodular worm.

No. 296. This Holstein male was born November 3, 1956, and inoculated February 16, 1957, with 100,700 *C. punctata* larvae. See figure 2 for resulting egg counts. Drops in egg counts occurred March 20, April 23, and November 1. No *C. punctata* eggs were recovered from this animal after November 1, even after reinoculation on November 7 with 45,545 larvae. Thus complete immunity was not established until after the third decline in egg counts. Details are omitted in figure 2 on 14 counts ranging from 9.2 to 2.5 EPG made between May 22 and July 11.

Nodular worm larvae (6,715) were included in the inoculation on November 7, and the first eggs were recovered on December 14, or 37 days later. The resulting egg counts are shown (fig. 2). This again indicated that immunity to *C. punctata* does not protect against nodular worm infection.

No. 298. This Jersey male was born December 18, 1956, and inoculated with 70,560 *C. punctata* larvae on February 18, 1957. See figure 2 for the resulting egg counts. Drops in egg counts occurred April 1 and September 11. No eggs were recovered from November 12 to December 19. In this animal, immunity was established following a second attempt. The animal was reinoculated on November



FIGURES 1–6. Egg counts during course of infection first with *Cooperia punctata* (Coop.) followed by *Oesophagostomum radiatum* (Nod.). Ordinates, eggs per gram of feces; abscissas, dates. Coop., *Cooperia punctata;* Nod., nodular worm, *Oesophagostomum radiatum;* Inoc., inoculation; Cts., egg counts. The prepatent period is indicated by a line extending to the right from the point of inoculation interpreted by the number of days.

7 with 45,545 *C. punctata* larvae. A very few eggs were recovered from December 10, or 33 days after reinoculation, until January 10, 1958. This is an instance of the appearance of *C. punctata* eggs at more than twice the normal length of the prepatent period.

Nodular worm larvae (6,715) were included in the inoculation of November 7, and on December 18, or 41 days after inoculation, the first nodular worm eggs were recovered. A range of 1.6 to 6 EPG was maintained until January 23, 1958 (fig. 2).

No. 311. This Holstein male was born September 3, 1957, and inoculated with 30,100 *C. punctata* larvae February 21, 1958. He received an equal amount of the same suspension of larvae as No. 312, to be described next. Because of the low count (fig. 3), he was reinoculated with 9,200 *C. punctata* larvae on May 20. For the results see figure 3. On August 11, the animal was again inoculated with 21,436 *C. punctata* larvae (not shown on fig. 3). Between August 17 and October 7 the counts did not exceed 0.7 EPG. These results seem to indicate that this animal already had a certain degree of immunity at the time of the first inoculation. This is especially indicated when compared with No. 312, the next animal to be described.

Nodular worm larvae (20,000) were included in the inoculation of May 20 and the first eggs were recovered on July 1. The resulting egg count is shown in figure 3 and indicates that this animal, although resistant to *C. punctata,* was susceptible to nodular worm infection.

No. 312. This Holstein male was born September 2, 1957, and inoculated February 21, 1958, with 30,100 *C. punctata* larvae on the same day as No. 311, with an equal amount of the same suspension of larvae. The resulting egg counts are shown in figure 3. He was reinoculated on June 30 with 25,000 *C. punctata* larvae; the results of the egg count were as indicated.

He was also inoculated with 79,420 nodular worm larvae on June 30, and the first eggs were recovered on August 11 (fig. 3). Between August 15 and October 24 the counts varied from 1.6 to 4.7 EPG and between October 27 and November 13 from 0 to 0.78 EPG.

No. 301. This Holstein male, born December 20, 1956, was inoculated with 45,545 *C. punctata* on November 7, 1957. The egg count results are shown in figure 4. He was reinoculated with 52,913 larvae on December 3, 1957, and 35,600 February 5, 1958. Beginning on March 21, or 44 days after the February 5th inoculation, there were 15 days of larger numbers with a maximum of 1.6 EPG. This suggests a retardation in larval development due to immunity. Between May 26, 1958, and January 26, 1959 (details not shown), there followed 69 counts that did not exceed 0.07 EPG, 51 of which were negative. That this animal probably possessed considerable immunity before the beginning of the series of three inoculations is indicated by the resulting low counts of *C. punctata* eggs in comparison with the results of No. 305, described next. This is further indicated by the fact that No. 301 received 4.5 times as many larvae as No. 305 (45,545 and 10,000, respectively). In addition, the larvae were from the same culture and from the same source.

Twenty-two thousand nodular worm larvae were included in the inoculation of February 5; the first eggs were recovered on March 25, or 48 days after inoculation (fig. 4). This indicated that immunity to *C. punctata* does not protect

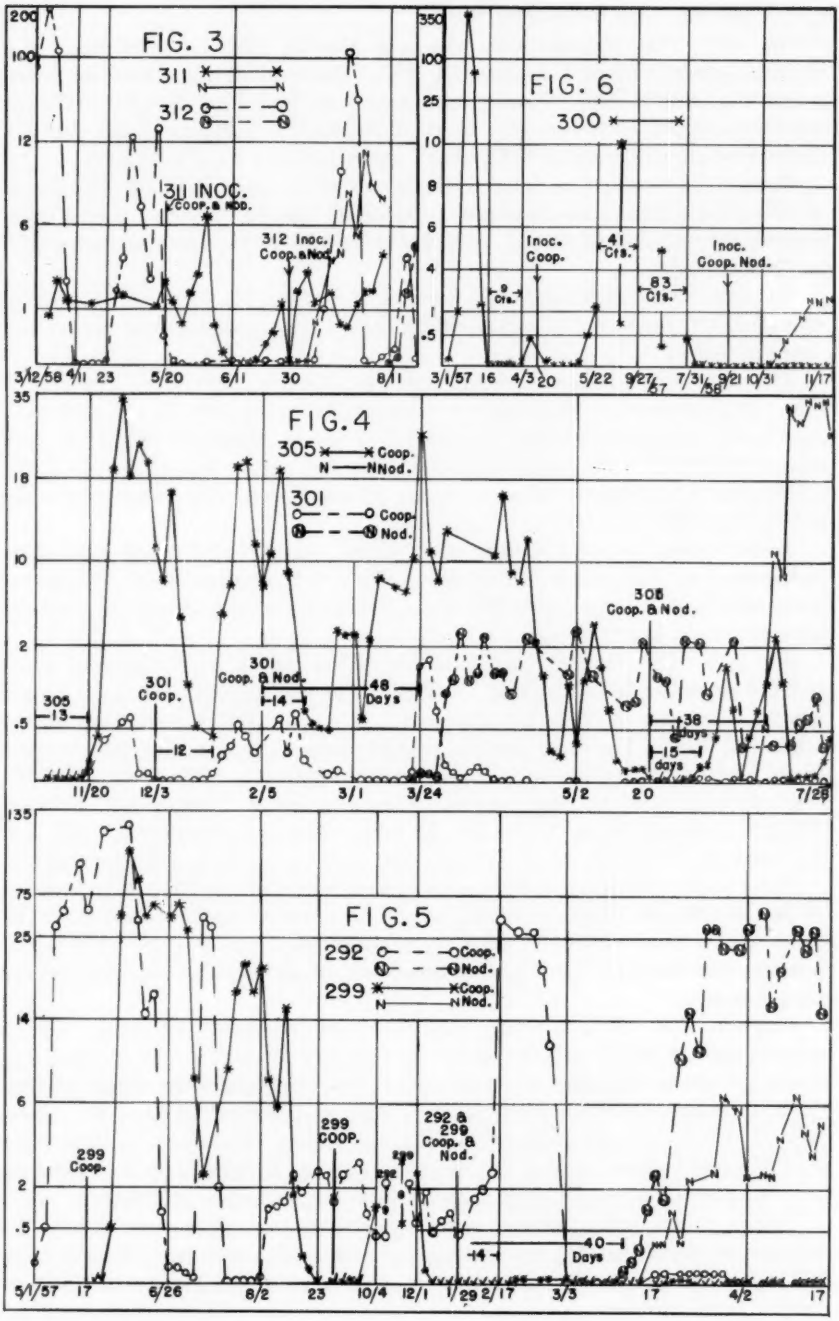


FIG. 3
311
312
311 INOC.
Coop. & Nod.
312 Inoc.
Coop. & Nod.
FIG. 6
300
Inoc.
Coop.
41
Cts.
9
Cts.
83
Cts.
Inoc.
Coop. Nod.
FIG. 4
305 Coop.
Nod.
301 Coop.
Nod.
305
13
301
Coop.
12
301
Coop. & Nod.
14
48
Days
305
Coop. & Nod.
38
Days
15
days
FIG. 5
292 Coop.
Nod.
299 Coop.
Nod.
299
Coop.
299
COOP.
292 &
299
Coop. &
Nod.
14
40
Days

against infection with the nodular worm.

No. 305. This Holstein male was born May 3, 1957, and inoculated with 10,000 *C. punctata* larvae November 13, 1957. The resulting egg counts are shown in figure 4. Fifteen days following reinoculation on May 20 with 9,200 larvae the egg counts varied as shown (fig. 4) until August 28, following which date six counts (not shown) made up to October 7 varied between 0 and 0.09 EPG.

Forty-eight thousand nodular worm larvae were included in the inoculation of May 20 and eggs were recovered as indicated (fig. 4). Twenty counts made between June 27 and October 7 yielded 0.11 to 43.7 EPG (last ones not shown in Fig. 4).

No. 292. This Holstein male was born November 11, 1956, and inoculated with 25,550 *C. punctata* larvae April 20, 1957. The resulting egg counts are shown in figure 5.

This animal was reinoculated with 35,600 *C. punctata* larvae on February 5, 1958. High counts occurred again between February 13 and 28, followed by a rapid decline to 0 between March 3 and 17. Between March 17 and 28, 40 days after reinoculation, a few eggs were recovered but always less than 0.09 EPG. Seventy-two examinations between March 31 and October 28 failed to produce any eggs definitely indentified as *C. punctata.*

Twenty-seven thousand nodular worm larvae were included in the inoculation on February 5. The nodular worm egg counts varied between 10 and 43 eggs between March 24 and April 16. This again indicated that immunity to *C. punctata* does not protect against infection with the nodular worm.

No. 299. This Holstein male was born November 3, 1956, and was given 91,400 *C. punctata* larvae on May 15, 1957. The resulting egg counts are shown in fig. 5. The egg count varied between 2.59 and 103 EPG between May 30 and August 12, 1957. The count quickly dropped to between 0 and 0.1 from August 21 to September 27. He was reinoculated August 27, 1957, with 12,000 *C. punctata;* eggs were recovered until December 3, as noted. This animal was given 97,500 *C. punctata* larvae on January 30, 1958. Between December 3, 1957, and November 10, 1959, only 11 *C. punctata* eggs were recovered in 136 examinations.

Ninety-seven thousand nodular worm larvae were included in the inoculation on January 30; the first eggs of this species were recovered on March 17. The counts varied between 1.8 and 6.8 EPG from March 21 to May 19. This again indicates that immunity to *C. punctata* does not afford protection against nodular worm infection.

Calf No. 300. This Holstein male was born November 19, 1956, and inoculated with 70,560 *C. punctata* larvae on February 18, 1957. The resulting egg counts are shown in figure 6. This animal was reinoculated on April 20 with 51,100 *C. punctata* larvae. The resulting counts are indicated in figure 6. About July 31, 1958, the eggs decreased rapidly and became 0. No *C. punctata* eggs were observed between August 2, 1958, and February 2, 1959, in 36 examinations, not even after reinoculation with 132,640 larvae on September 21, 1958.

Fifteen thousand five hundred nodular worm larvae were included in the inoculation of September 21; the first eggs of this species were recovered October 31, (fig. 6) indicating that immunity to *C. punctata* does not protect against nodular worm infection.

TABLE I. *Results for 19 additional animals.*

Animal No.	First egg count EPG	First egg count Time (days)	Second egg count EPG	Second egg count Time (days)	Third egg count EPG	Third egg count Time (days)	Reinoculation
270	2.1–36	600	*1.24–34	180	—	—	*10 neg. counts .35 days **fig. 10
272	1.37–133.0	119	*4.5–102	66 (1)	.64–4.7*	26	6 neg. counts in 8 days
275	1.68–100	640	.01–.11	6 (2)	—	—	12 neg. counts over *35 days **fig. 1C
278	0–.73	62	—	—	—	—	Inoc. 3 times 41 and 25 days apart. Natural immunity?
279	12–27	92	*1.7–18	17	—	—	Dropped to 0
280	4.1–105	447 (4)	—	—	—	—	9 days neg. *(3) **fig. 6
281 A	max. 74	13	max. 3.7	19	max. 4.4	6	*3 times natural immunity
282	.46–1.61	33	1.2–14.7	38	—	—	neg. 3 counts 3 days
283	124–1050 (5)	33	.49–20.5	35	—	—	***Dropped to 0
284	.95–63	36	.42– 2.5	172	—	—	*(6)
285	17.5–141.8 (7)	143	.42–14.3	922	—	—	***
288	83–1845	41	—	—	—	—	***Dropped to 0 15 days
290	3.5–45.6 (8)	139	.33–3.57	901	—	—	*** **see fig. 11
293	5.2–23.6	29 (9)	.38–.98 (10)	65	*.56–4.4	334	*(11)
294	19.9–145.8	135 (12)	—	—	—	—	*(12)
295	.18–4.6	200 (13)	—	—	—	—	*(13) **fig. 3
302	326 max.	14 (14)	max. 31.9	15 (15)	1.3–4.8	133	***Dropped to 0–.17 for 99 days (16)
303	.05–1.68	27 (17)	.88–38.5*	27 (18)	.08–.35	34 (19)	*0–.08 E.P.G.
310	0–.03	63	—	—	—	—	*Twice, result 0.04–0.78 natural immunity?

* Reinoculated. ** See Mayhew et al, 1959. *** Not reinoculated.

(1) Dropped to 0 for 14 days.
(2) Followed by 13 negative counts for 34 days before reinoculation.
(3) 0.11 to 0.34 EPG in 18 counts over 36 days after reinoculation.
(4) 205 counts.
(5) Dropped to 0 in 10 days.
(6) Dropped to 0 for between 6 and 10 days followed by 0 to 0.23 EPG.
(7) Dropped to 0 for 8 days.
(8) Max. and min. within 4 days of each other. Three months later dropped to 0.31 to 1.21 EPG for 9 days, then gradually increased to the level of the second egg count period.
(9) Negative at least 8 days.
(10) Dropped to zero 12 days after reinoculation, and was zero for 6 days. Third egg count followed which dropped to 0.
(11) Reinoculated twice after 0 for at least 9 days. Result max. of 0.14 EPG. As a result of being inoculated with nodular worm larvae he attained a maximum of 0.31 EPG.
(12) Negative 12 days (11 examinations) counts between 0.01 and 0.12 EPG for 13 days. After reinoculation was negative for 11 days followed by counts 0 to .05 EPG for 42 days.
(13) Dropped to between 0 and 0.10 EPG for at least 24 days. Following reinoculation five counts between the 9th and 15th days reached a maximum of 0.57 EPG followed by five negative counts during the next 13 days.
(14) Dropped to 0.
(15) Dropped to four negative counts in 6 days.
(16) No. 302 was inoculated with nodular worm larvae at the same time as with *Cooperia* larvae. He maintained a count of between 4.15 to 27.8 EPG for 194 days, then dropped rapidly to between 0.01 and 0.38 EPG for 62 days.
(17) Dropped to 0.
(18) Dropped to 0 for 11 days; four counts.
(19) Dropped to 0. This animal was given nodular worm larvae at the time as the two reinoculations with *Cooperia* with the resulting egg count of 0 to 0.25 EPG.

Retardation of Development of Larvae following Reinoculation

It has been noted that an unusually long period of time elapsed between reinoculation and an increase in the egg count level in some animals. This suggests the possibility that immunity had retarded the development of larvae in these instances. As a matter of information we are presenting these figures: Calf No. 298, 33 days elapsed between reinoculation and the reappearance of eggs in the manure; No. 311, 32 days; No. 301, 44 days; No. 292, 40 days; No. 284, 24 days; No. 293, 29 days; No. 299, between 31 and 37 days after first reinoculation and 20 after the second.

On the other hand No. 281B had an increase in egg counts on the 16th day, No. 297 on the 17th, and No. 295 had two low counts only on two successive days on the 12th and 13th days after reinoculation.

The prepatent period, according to our data, is between 9 and 17 days.

The number of days between the drops and increases in egg counts associated with the establishment of or attempts to establish immunity varies between 4 and 15 days as follows: No. 281B, 4 and 9 days; No. 297, 15 days; No. 296, 7 and 8 days; No. 298, 10 days; No. 312 between 8 and 13 days; No. 305, 7 and 7 days; No. 292, 9 and between 7 and 15 days; No. 283, about 9 days; No. 284, 7 days; No. 285, 9 days; No. 290, 10 days; No. 295, 10 days. These considerably lower figures suggest that the delayed increases in egg counts are not due to the recovery of egg production by females of former inoculations.

SUMMARY

Results are reported of studies on 30 animals inoculated with *Cooperia punctata* which showed evidence of the development of an immunity to the parasite. Egg counts were used as a measure of the immunity.

Twenty-six of these animals were reinoculated with no rise in egg counts, or only a short period of increased counts resulting.

The first indication of immunity was a rapid fall in egg count numbers. The counts may increase for a period following low or negative counts, then drop and increase again; this phenomenon occurred as many as three times before remaining low or negative.

Some animals showed evidence of a degree of resistance to infection with the first inoculation. (Nos. 278, 281A, 310, and 301).

The longer prepatent period found in some animals after reinoculation, as much as two to three times the normal, gave evidence of retardation of larval development.

Eleven animals were inoculated with nodular worm larvae (*Oesophagostomum radiatum*) after they had shown evidence of immunity to *Cooperia punctata* with the result that all became infected.

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THE ACTION OF PHENOTHIAZINE ON THE METABOLISM OF INFECTIVE NEMATODE LARVAE*

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Relatively little information is available concerning the metabolism of parasitic nematode larvae. Evidence of the functioning of the Krebs' tricarboxylic acid cycle in certain of the parasitic nematodes has been found by Costello and Grollman (1959) and by Massey and Rogers (1949). Rogers (1948) found a Qo_2 (microliters of oxygen uptake per hour per milligram dry weight) of 18.4 for young *Nippostrongylus muris* larvae. A similar value was found for third stage *Haemonchus contortus* larvae. Jones et al (1955) demonstrated the occurrence of high energy phosphate compounds in filariform larvae of *Strongyloides ratti.*

More research has been done with adult forms. Evidence has been presented (Rogers, 1949b) that trichostrongyles utilize aerobic pathways. Massey and Rogers (1949) were able to increase the oxygen uptake of adult worms by adding Krebs' cycle intermediates. However, a relative insensitivity to malonate inhibition was found. Rogers also increased the uptake by adding cytochrome c to the media. However, another group (Bueding and Charms 1952) were unable to find either cytochrome c or cytochrome oxidase in *Ascaris lumbricoides* or *Litomosoides carinii.* Rogers (1949a) demonstrated the presence of hemoglobin in *Haemonchus contortus* and *Nematodirus spp.* but pointed out its inefficiency as an oxygen carrier at low oxygen tensions.

Work relevant to the site of action of anthelminthics on the parasite has revealed at least two types of action. One group of anthelminthics consists of proteolytic enzymes which destroy the nematodes by digesting their tissues. The second group inhibit certain metabolic reactions. Piperazine paralyzes *Ascaris lumbricoides* by blocking and neuromuscular junction (Norton and DeBeer, 1957). Phenothiazine inhibits succinoxidase in rat liver mitochondria at a concentration of 1.4×10^{-5} M (Collier and Allenby, 1952) and human or rabbit erythrocyte glyoxalase at a concentration of 10^{-6} M (Collier and MacRae, 1953). These same workers (Collier, 1940, Collier and Allen, 1942a, 1942b, Allenby and Collier, 1952) have shown that the oxidized derivatives of phenothiazine are more efficient enzyme inhibitors. Harpur et al (1950) found that phenothiazine was not oxidized in the rumen or any location oral to the bile duct. Esserman (1952) has also presented evidence that the anthelminthic activity of phenothiazine is due to the parent compound.

This study was instigated to investigate the site of action of phenothiazine on third stage infective nematode larvae cultured from ovine feces. The phenothiazine used in these experiments was furnished as N.F. purified by E.I. DuPont de Nemours & Co., Wilmington, Delaware.

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MATERIALS AND METHODS

Larval Culture

Infective larvae were obtained from naturally or artificially infected animals by culturing feces for 7 days. Feces were collected from animals on a 24-hour basis during the period in which the nematodes had the maximum ova output. The feces were slightly dried to facilitate macerating the material, mixed with granulated bone charcoal, moistened, and placed in quart jars. After storage at room temperature in the dark for 7 days, the material was placed in a Baermann apparatus and the larvae allowed to migrate for 6 to 8 hours. The larvae were then washed several times by decantation in tap water until the supernatant solution was clear. If the larvae were contaminated with charcoal or debris, they were filtered and resuspended in the Baermann to clarify. Clarified larvae were suspended in isotonic sodium chloride solution containing 2 g of streptomycin per liter, 2,000,000 units of penicillin G per liter, and 200,000 units of mycostatin per liter. These larvae were maintained at 10 C until required for experiment, usually a period of 2 to 5 days. Before use in the experiment, the larvae were washed three times with Krebs-Ringer-phosphate buffer mixture. These larvae were also used to continue the infection in 4- to 5-month-old wethers. The third-stage larvae from each batch cultured were examined and identified. When larvae were obtained from mixed infections, the following was a typical distribution: *Chabertia ovina,* 27 percent; *Trichostrongylus spp.,* 42 percent; *Cooperia spp.,* 11 percent; *Osteragia spp.,* 6 percent; *Oesphagostomum spp.,* 4 percent; unidentified, 10 percent. Larvae obtained from a relatively pure *Haemonchus* infection proved to be 94 to 98 percent *Haemonchus placei.*

Phenothiazine Determination

The level of phenothiazine in feces was determined by a modification of the method of DeEds and Eddy (1937). A measured quantity of dried feces is extracted with hot ethanol, the extract is cooled, brought to constant volume, and an aliquot is removed. One-fifth volume of bromine solution is added to the aliquot and the excess bromine vaporized off; the solution is cooled and read in a colorimeter at 5050 A against a blank prepared from feces containing no phenothiazine.

In Vitro Embryonation and Survival Studies

Studies on embryonation and larval survival were made in small petri dishes containing the substances to be studied. All dishes were adjusted to constant volume with isotonic saline. Cultures were examined with a stereoptic microscope or a conventional binocular microscope. As infectivity was not determined for these worms, they were arbitrarily segregated as to straight and inactive (dead), or coiled and active forms.

Respiration Studies

Oxygen uptake was determined using a circular type Warburg respirometer employing flasks of approximately 7 ml volume. The technique of Umbreit et al (1957) using air as the gas phase was followed in all of these studies. Previous work in this laboratory had indicated that oxygen offers no advantage as the gas phase. The manometric readings were plotted vs time and the microliters of oxygen calculated from the slope of the initial uptake. Qo_2 was calculated using dry-weight measurements and $Qo_2/10^5$ larvae was calculated using larval counts (counts were made separately for each experiment).

In some experiments, carbon 14-labelled substrates were added in order to measure $C^{14}O_2$ production. In these experiments, the radioactive CO_2 was trapped by KOH in the center well of the Warburg flask, precipitated with $BaCl_2$, plated by filtration on to filter papers, and counted in a Nuclear, gas flow, ultrathin window counter. Counts were corrected to infinite thinness and also corrected to 10^5 larvae. Specific activity, though calculated, is not reported because much of the center well carbon was absorbed from the atmosphere during flask equilibration.

Methylene Blue Reduction Experiments

The Thunberg technique (Umbreit, 1957) was used for the estimation of dehydrogenase activity. Colorimetric readings of methylene blue reduction were made at 6600 A using a "Spectronic 20" spectrophotometer. The logarithm of the optical density was plotted vs time and the best straight line drawn to determine the rate of methylene blue reduction.

RESULTS

Embryonation and Hatching of Nematode Eggs in Phenothiazine Suspension

Eggs cultured from ovine feces were suspended in saline, bile (1:50), phenothiazine (0.003 M), and bile plus phenothiazine. Those suspended in saline or bile solutions embryonated and hatched but eggs did not embryonate in either pheno-

thiazine solution. Eggs from sheep feces were placed in saline, phenothiazine (0.003 and 0.006 M), or extracted feces from sheep on phenothiazine-salt mixture of various phenothiazine concentrations (feces contained 0.0013, 0.0021 or 0.0034 M phenothiazine on a dry weight basis). Embryonation was inhibited at all concentrations tested. Hatching was almost completely inhibited at 0.003 M phenothiazine (either purified or from feces) but only partially inhibited by feces containing 0.0013 M phenothiazine (25 percent larvae at fourth day compared with 56 percent for the control).

Survival of Larvae in Phenothiazine Suspension in Vitro

Eight-day-old nematode larvae cultured from ovine feces were suspended in saline, saline + phenothiazine (0.0125 M), saline + bile (1 : 50), and saline + phenothiazine + bile. The percentage straight or inactive larvae increased from a control level of 14 percent to 44 percent when phenothiazine was present and the activity of the remaining larvae was greatly diminished. Bile had no apparent effect, either by itself or as an adjuvant to phenothiazine. When various concentrations of phenothiazine were used, it was found that when the percentage dead larvae were plotted vs. phenothiazine concentration, a plateau was reached at approximately 0.006 M. Also, the greatest rate of kill occurred during the first 3 days of incubation.

Effect of Phenothiazine on Oxygen Uptake of Nematode Larvae

In the first series of experiments, 7-day-old mixed nematode larvae from sheep feces were homogenized in isotonic potassium chloride and the respiration measured in various media with and without the addition of phenothiazine. Very low oxygen uptakes were obtained with homogenates but phenothiazine apparently did not inhibit oxygen uptake in this preparation.

In experiments using whole larvae, 0.5 ml of larval suspension was employed per flask; this comprised about 35,000 larvae. A concentration of 0.005 M phenothiazine was used throughout. Because the mixed cultures varied in number of species, amount of each species, and viability of the culture, Qo_2 values were only comparable within experiments. However, oxygen uptake was consistently inhibited with either pyruvate or glucose as the substrate (30 micromoles of substrate per flask) when phenothiazine was added. This inhibition amounted to an average of 38.7 percent (100 percent—Qo_2 phenothiazine per Qo_2 control) for pyruvate and 41.8 percent for glucose. Duplicate flasks were measured for each experiment and five experiments were carried out. In three experiments using fumarate as the substrate, phenothiazine was inhibitory in two, but an apparent stimulation was noted in the third. One experiment was run using succinate as the substrate; an inhibition of 56.4 percent was found.

In an attempt to obtain more consistent uptakes, sheep were infected with *Haemonchus placei*. In the first experiment conducted with these cultures, 0.05 micromole of methylene blue was added to one replicate. This stimulated oxygen uptake both with and without phenothiazine and was included in all the remaining experiments. The *Haemonchus placei* results are summarized in table I.

TABLE I. *The Effect of phenothiazine on the oxygen uptake and radioactive carbon dioxide production by* Haemonchus placei *in vitro.*

Substrate	Treatment	Q_{O_2}	$Q_{O_2}/10^5$ larvae	Cpm/10^5 larvae
———	Control	3.745	25.66	—
———	Phenothiazine, 5 micromoles	2.812	19.12	—
Glucose, 30 micromoles	Control	7.810	40.79	—
" " "	Phenothiazine, 5 micromoles	4.709	25.08	—
Pyruvate, 30 micromoles	Control	7.898	41.43	—
" " "	Phenothiazine, 5 micromoles	5.429	28.68	—
α-ketoglutaric acid, 6 micromoles	Control	9.467	70.70	4248.6
" " "	Phenothiazine, 5 micromoles	5.052	38.40	569.0
Succinic Acid, 30 micromoles	Control	8.177	62.13	605.3
" " "	Phenothiazine, 5 micromoles	6.143	46.61	398.8
Fumaric Acid, 30 micromoles	Control	9.387	70.24	404.8
" " "	Phenothiazine, 5 micromoles	5.306	40.87	601.5

Each figure represents an average of two replicates with duplicate flasks per replicate.

Effect of Phenothiazine on Radioactive Carbon Dioxide Production from Labelled Substrate

The amount of radioactive carbon found in the center well of the Warburg flask at the conclusion of a routine manometric experiment in which C^{14}-labelled substrates were used is a fair estimate of the substrate utilization. Since nonradioactive CO_2 is absorbed by the center well during equilibration, specific activity calculations were not used. The total counts per minute minus the background count were corrected to infinite thinness by standard methods and adjusted to counts per minute (cpm) per 10^5 larvae. In three experiments in which mixed nematode larvae were used, considerable variability was encountered. The addition of radioactive pyruvate (pyruvate-3-C^{14}) as a substrate resulted in more labelled CO_2 production than when radioactive glucose (uniformly labelled) was added. Phenothiazine definitely inhibited labelled CO_2 production in either case. This inhibition (using the same formula as given in the last section) amounted to 65.4 percent when pyruvate was the substrate and 43.9 percent when glucose was the substrate. When the "*t*" test for significance (Snedecor, 1946) was applied to the mean differences, this inhibition proved to be highly significant in both cases ($P < .01$). For the experiments in which the pure *H. placei* culture was used, sodium succinic-1-C^{14} acid, α-ketoglutaric acid-5-C^{14} and fumaric-1-C^{14} acid were used as substrates. These results are given in table I.

Effect of Phenothiazine on Methylene Blue Reduction

In these experiments, phenothiazine was added to Thunberg tubes containing methylene blue, Krebs-Ringer-phosphate buffer, and either sodium succinate or α-ketoglutaric acid. The tubes were evacuated, incubated at 37 C and the concentration of leuco-methylene blue measured at various intervals. In an initial experiment, the number of larvae was varied in an attempt to determine the optimum concentration. It appeared that 0.75 ml of larvae (about 16,000) gave the maximum rate of reduction. In subsequent experiments, 0.5 ml of larvae suspension was employed. The number of larvae varied considerably between runs. Since the previous experiment had indicated that the rate of reduction was related to the number of larvae when this number was kept below 2×10^4, the data were adjusted to a uniform number of larvae. The results of this experiment are given in table II.

TABLE II. *The effect of phenothiazine on the rate of reduction of methylene blue by nematode larvae with α-ketoglutaric acid or sodium succinate as the substrate.*

	Control	0.005 millimole phenothiazine
Sodium succinate		
0.025 millimoles	3.70* (2)**	
0.050	2.92 (3)	2.57 (12)
0.075	1.95 (2)	
α-ketoglutaric acid		
0.025	4.15 (2)	
0.050	2.67 (2)	1.36 (4)
0.075	2.45 (2)	
0.100	0.88 (2)	

* = 2 + log (log Io/I) per minute per 10^7 larvae.
** Number of experiments given in parentheses.

DISCUSSION

Our experiences with nematode larvae during these experiments has led to the conclusion that, to approach consistent results between experiments, it is necessary to establish a relatively pure infection. However, phenothiazine inhibited larval metabolism in all the experiments conducted.

Because phenothiazine is absorbed through the cuticle rather than ingested (Esserman and Sambell, 1951), bile was added to the substrate media in an attempt to improve absorption. According to our results, bile did not improve the ability of phenothiazine to inhibit egg embryonation or hatching, nor did it increase the rate of kill of larvae by phenothiazine in vitro.

A few experiments were attempted using larvae homogenates in hopes that by comparison with whole larvae results, we could elucidate the importance of the cuticle and determine whether phenothiazine inhibition is related to substrate transfer through membranes. We were unable to obtain sufficient oxygen uptake using homogenates to justify conclusions with respect to this problem. Further work is progressing with homogenates in hopes that by using a more sensitive method, we can resolve this problem.

Further investigation will be necessary to determine the site of action of phenothiazine. In these experiments, phenothiazine inhibited the reduction of methylene blue when α-ketoglutaric acid was used as the substrate. This observation was confirmed by CO_2 production trials using labelled acid. The reduction of methylene blue by larvae in the presence of α-ketoglutaric acid diminished as the substrate concentration was increased. Some substrate inhibition of methylene blue reduction was observed with succinate but very little inhibition occurred with the addition of phenothiazine. However, using labelled succinic acid, very little carbon^{14} dioxide production was observed; this was also true when labelled fumaric acid was used. These data indicate that phenothiazine inhibits either a decarboxylase or dehydrogenase system involved in the conversion of α-ketoglutaric acid to succinic acid. The apparent lack of utilization of labelled succinic and fumaric acids by these larvae is presently being more thoroughly investigated using other methods.

SUMMARY

1. Embryonation of nematode eggs was prevented by phenothiazine at a concentration of 0.0013 M in the feces (dry weight). Hatching of embryonated eggs was almost completely inhibited at 0.003 M phenothiazine.
2. Phenothiazine absorption into the larvae, as measured by dead (inactive) larvae,

was not increased by bile.

3. Phenothiazine inhibited the oxygen uptake of either mixed nematode larvae or *Haemonchus placei* larvae. This inhibition amounted to between 20 and 40 percent and was found either in the absence of added substrate or when glucose, pyruvate, α-ketoglutaric acid, succinic acid, or fumaric acid was added to the incubation media.
4. Phenothiazine inhibited the production of radioactive CO_2 when labelled glucose or pyruvate substrates were used by mixed nematode larvae and when labelled α-ketoglutaric acid was used by *H. placei* larvae. When labelled succinic or fumaric acid were added to *H. placei* larvae, only a slight amount of radioactive CO_2 could be detected in the center well.
5. Phenothiazine inhibited the rate of methylene blue reduction by mixed nematode larvae when α-ketoglutaric acid was added as the substrate, but not when succinic acid was added.

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A NEW DIHYDROTRIAZINE EFFECTIVE AGAINST *SYPHACIA OBVELATA* IN MICE

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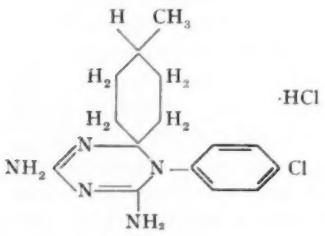
Various piperazine compounds show excellent activity against *Syphacia obvelata* in mice (Mouriquand, et al, 1951; Brown, et al, 1954) and Brown and his coworkers have found that, in general, compounds active against *Syphacia* were also active against the human pinworm, *Enterobius vermicularis*. As the piperazines are more effective against mature pinworms than against immature forms, it is necessary to give repeated doses in order to eliminate the worms as they mature.

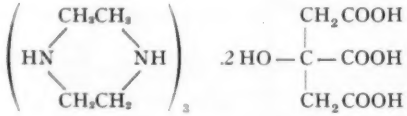
Several years ago a number of dihydrotriazines were given us for anthelmintic screening and were found active against *Syphacia*. Since then Roth (Roth, et al, 1960) has prepared a large number of these compounds, all of which showed some pinworm activity in single doses. One compound, B.W. 58-232, was found to be among the most active and, at the same time, the least toxic of the series. The oral LD_{50} of this compound was determined by Mr. Rocco Fanelli, of this Laboratory, who found that 20 g per kg of body weight failed to kill any of the mice receiving this dose.

A comparative study of the activity of B.W. 58-232 and tripiperazine dicitrate, the salt of piperazine used in Antepar®, in single doses at various dosage levels was undertaken. The structures of the two compounds are:

B.W. 58–232



Tripiperazine dicitrate



PROCEDURE

Each mouse was kept in a small, individual cage during the course of the experiment. In each cage was a feeding rack, designed to prevent food particles from falling through the floor of the cage; attached to each cage was a water bottle; and beneath each cage was a pan of water to collect feces and worms passed by the mouse.

B.W. 58–232 was ground in a mortar with 1 to 3 drops of Tween 80, water added a little at a time during grinding until the required amount of water was used and the resulting emulsion was ready for dosing the mice. As piperazine citrate is quite soluble in water, it was dissolved directly in the required amount of water. Prior to dosing, each mouse was weighed, the proper amount of emulsion or solution drawn into a 1 ml syringe, graduated in hundredths, the blunt needle inserted into the stomach, and the dose administered.

At the end of 24 hours, the pan under each cage was removed and another pan of water substituted for it. The contents of each pan was poured, a little at a time, into a petri dish, the dish placed under a dissecting microscope, and all worms counted. The worms were differentiated

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into mature and immature. At the end of 48 hours the mouse was autopsied, the cecum and large intestine removed, opened, and all material washed out. The number of worms remaining was tabulated. Then the pans from the second 24 hours were examined and all worms counted.

This procedure, using naturally infected mice, is somewhat slower, but more accurate, than the customary one of comparing the number of worms left in treated animals with the number remaining in control animals, both groups being artificially infected with, in theory, approximately the same number of mature eggs. This method enables one to determine the percentage of worms passed during each 24-hour period, the percentage remaining at autopsy, the relative numbers of mature and immature worms, whether the compound is more effective against one age of worm than another, and whether a compound has a short or long period of activity in the intestine.

To determine whether mice would accept this drug in food, some fox chow was sifted through a 40-mesh sieve, the sifted chow thoroughly mixed with the desired ratio of drug, the whole slightly moistened with water, and placed in small dishes in the individual cages for 24 hours. Then the drug-chow mixture was removed and the regular chow substituted. Worms were collected in the pans below the cages and counts made of worms passed, as well as worms left at autopsy.

RESULTS

The results of the trials of both compounds at the different dosage levels are summarized in table I and figure 1. At each of the 4 higher dosage levels, 300 to 600 mg per kg, B.W. 58-232 gave higher percentages of clearance of all worms, as well as clearing a much larger proportion of the mice. At the two lower levels, piperazine citrate gave better elimination of worms, but these lower levels are not sufficiently effective for practical purposes.

B.W. 58-232 showed somewhat comparable activity against both mature and immature forms, whereas piperazine citrate was much more effective against mature worms, which is best illustrated in figure 1. Piperazine was more variable in its

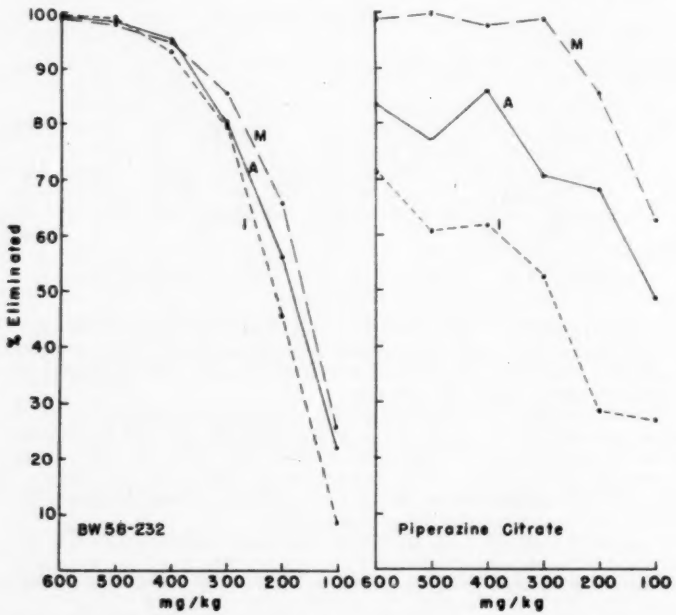

FIGURE 1. *Syphacia obvelata* eliminated at various dosage levels. A, all worms; I, immature worms; M, mature worms.

activity in single doses, due to different percentages of mature to immature worms in naturally infected mice.

TABLE I. Syphacia obvelata *eliminated at various dosage levels.*

Dosage mg/kg	B.W. 58–232			Piperazine citrate		
	Av. no. worms per mouse	Av. percentage eliminated	Cleared / treated	Av. no. worms per mouse	Av. percentage eliminated	Cleared / treated
			ALL WORMS			
600	77.6	99.1	16 : 20	75.3	83.8	5 : 15
500	61.9	98.7	15 : 20	60.9	77.2	5 : 16
400	78.1	95.2	16 : 20	62.1	86.1	4 : 16
300	54.5	79.6	8 : 20	99.5	70.4	1 : 15
200	62.8	56.2	6 : 20	75.0	68.1	0 : 16
100	64.7	22.0	0 : 20	81.3	48.6	0 : 15
			IMMATURE WORMS			
600	14.0	99.6	18 : 19	42.0	71.3	5 : 15
500	27.0	99.2	15 : 17	26.5	60.7	5 : 16
400	34.1	93.3	14 : 17	21.4	61.7	4 : 16
300	17.0	85.8	11 : 17	62.3	52.3	1 : 15
200	23.1	45.7	7 : 17	22.4	28.7	1 : 15
100	22.5	8.3	0 : 19	28.7	27.1	1 : 15
			MATURE WORMS			
600	64.4	99.0	16 : 20	33.3	98.9	12 : 15
500	34.9	98.0	16 : 20	34.4	100.0	16 : 16
400	49.2	94.9	16 : 20	40.6	97.8	13 : 16
300	40.1	79.7	8 : 20	37.1	98.9	12 : 15
200	43.2	65.9	7 : 20	52.6	85.3	6 : 16
100	43.3	25.5	1 : 20	52.6	62.6	2 : 15

The percentages of *Syphacia* eliminated during 24 and 48 hours are shown in figure 2. The soluble piperazine citrate eliminated practically all of the affected worms during the first 24 hours and few were passed between 24 and 48 hours, thus producing curves that almost leveled off after 24 hours. On the other hand, the practically insoluble B.W. 58-232 continued to exert activity after 24 hours and the curves showed definite increases between 24 and 48 hours. Of the 47 mice receiving the three higher dosage levels, 400 to 600 mg per kg of piperazine citrate, only nine, or 19 percent, passed additional worms during the second 24 hours, whereas 45, or 75 percent, of the 60 receiving B.W. 58-232 at these same levels continued to pass worms during the second 24 hours.

Ten mice received fox chow with B.W. 58-232 added to it at the rate of 20 mg of compound per g of chow. The mixture was left in each cage for 24 hours, removed and the regular chow substituted. The mice passed from 24 to 86 *Syphacia* each, with an average of 42.8 worms, and all were negative at autopsy. No accurate determinations of amount of food and drug consumed were made, as the mice scattered much of the mixture, which fell into the pans of water beneath the cages. Further trials will be made, with varying ratios of drug to food and with the mixture molded into cakes and dried.

DISCUSSION

Although piperazine compounds have, in recent years, been the most effective and least toxic of anti-pinworm remedies, it is necessary to give a series of doses in order to eliminate all worms. A 1-day course of treatment with a compound having no more toxicity than piperazine citrate, which has an oral LD_{50} in mice of about 13.5 g per kg, would be a desirable addition as a therapeutic agent.

B.W. 58-232 is much less toxic than piperazine citrate, for it has an oral LD_{50} in mice of over 20 gms/kg. In comparative low-level trials with single doses it proved

to be superior to piperazine citrate, in that it eliminates both mature and immature worms about equally and causes a loss of pinworms over a longer period of time.

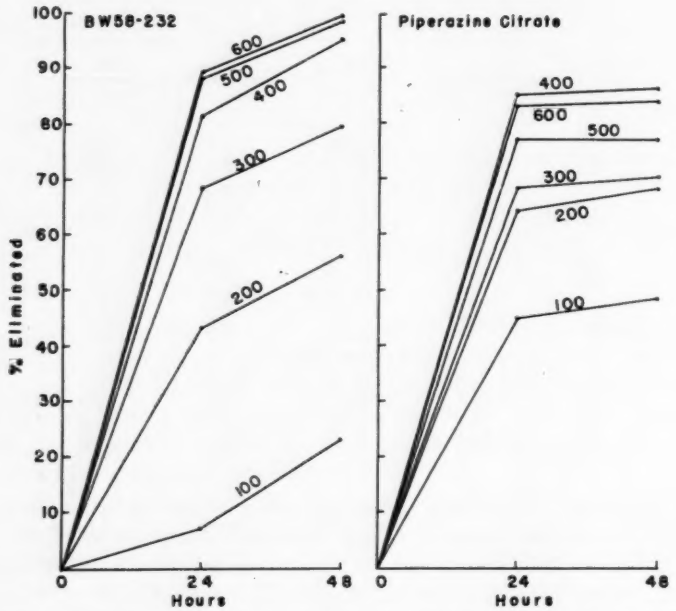

FIGURE 2. Percentages of *Syphacia obvelata* eliminated during one and two days at various dosage levels. The figures adjacent to the lines refer to the number of mg per kg received.

As the relatively insoluble B.W. 58-232 can be mixed with food and retain its activity, it may offer a more practical means of freeing colonies of mice from *Syphacia* infections.

SUMMARY

1. Comparative trials were made of the effectiveness of a new dihydrotriazine, B.W. 58-232, and of piperazine citrate in single doses against *Syphacia obvelata* in mice.

2. B.W. 58-232 proved superior in that: (a) it is less toxic; (b) it eliminates both mature and immature stages; (c) it clears a larger percentage of mice; (d) it causes a loss of worms over a longer period of time; and (e) it is effective when mixed with food.

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EXPERIMENTAL STUDY OF *DERMACENTOR MARGINATUS* SULZ. AND *RHIPICEPHALUS ROSSICUS* JAK. ET K. JAK. TICKS AS VECTORS OF TULAREMIA*

V. G. Petrov

The importance of the ixodial ticks as vectors of tularemia has been noted by many investigators in the Soviet Union and abroad.

D. A. Golov (1934 and 1935) and D. A. Golov and V. N. Fedorov (1934) showed experimentally the capacity of *Dermacentor marginatus* larvae infected with the tularemia organism to transmit the infection to rodents and domestic animals in the nymph or in the sexually-mature tick stage. Four nymphs were the minimum number of specimens used for transmitting the infection to a rodent. Transfer of tularemia to domestic animals was accomplished by attaching a few score adult infected ticks to them.

R. Ya. Chernina (1953) also obtained positive results in transmitting tularemia to animals by infected nymphs and sexually-mature ticks of *D. marginatus.*

Ya. F. Shatas and N. A. Bystrova (1954) repeatedly found the *D. marginatus* and *Rhipicephalus rossicus* ticks to be spontaneous carriers of the tularemia organism; also, they were the first to establish the occurrence of spontaneous infection in the latter species of tick. They proved experimentally that *Rh. rossicus* ticks, when infected in the larval phase, preserve the tularemia pathogens during their subsequent development into nymphs and sexually-mature ticks. The above authors succeeded in transmitting the infection to a white mouse by means of 3 or more such nymphs.

These same authors established presence of the infecting organism in excreta of nymphs and adult ticks developed from infected larvae.

In these investigations, however, it was not made clear that a single nymph or a single sexually-mature tick can transmit the disease, a fact very important in the evaluation of the significance of this vector. In the experiments of Ya. F. Shatas and N. A. Bystrova with *Rh. rossicus* the ability of the sexually-mature ticks to transmit the tularemic infection when feeding on susceptible animals remained unproven.

None of the authors listed studied the problem of possible transmission of the infection by larvae during interrupted feeding. Finally, the question of growth of tularemia bacteria in bodies of the species of ticks mentioned, during the course of their metamorphosis, was not clarified. All this stimulated us to undertake the investigations, the results of which are presented below.

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* Translation from the Russian for the Russian Translation Program, Division of Research Grants, National Institutes of Health, by the U. S. Joint Publications Research Service, New York.

Note by the Editor: This translated article by V. G. Petrov is from *Voprosy Epidemiologii I Profilaktiki Tuliaremii* (Problems of Epidemiology and Prophylaxis of Tularemia), Medgiz, 1958, Moscow (book edited by N. G. Olsuf'ev et al.) It is published here at the suggestion of a recognized worker in the field who wrote me that it has some obviously new data on the vector relationship.

D. marginatus and *Rh. rossicus* ticks, bred from uninfected female ticks, were used in the experiments.

The ticks were bred in the laboratory according to the method of N. G. Olsuf'yev (1941). Fed ticks in all stages of satiety were placed in test-tubes containing moistened pine shavings and kept at a temperature of 23 to 27 C. Under these conditions, the female ticks which had sucked their fill laid eggs, and the latter completed their embryonic development. Ten to 20 days after hatching from the eggs the larvae were attached to animals and allowed to feed. Nymphs were allowed to feed 1 or 2 weeks or longer after molting from the satiated larvae. Readiness of nymphs to suck blood was determined by change in color of their intestine resulting from the elimination of dark masses of digested blood. The sexually-mature ticks were fed no sooner than 1 to 2 weeks after molting from the nymph stage. The larvae and nymphs were applied freely to the animals (that is, they were not taped down). To keep them from being thrown off, small collars made of photographic film were put on the animals. The adult ticks were placed in glass cylinders which were taped onto the shaved back of a guinea pig.

Hungry female and male *D. marginatus* ticks and unfed larvae, nymphs, and sexually-mature *Rh. rossicus* ticks were put into a refrigerator at 3 C, two weeks after molting, whereby their life was prolonged. To forestall egg-laying the fed female ticks of both species also were kept in the refrigerator.

The following "first-group" animals (according to the classification of N. G. Olsuf'yev and T. D. Dunayeva) were used as sources (donors) for infecting the tick larvae: the hamster, common field vole, and steppe lemming. Infection of these animals was accomplished by subcutaneous injection into the groin of 10 cells of a virulent strain of tularemia bacteria, or by the prick of a needle infected with the spleen of an animal which had died of tularemia. We utilized the virulent strain No. 503, which had been isolated from *Dermacentor pictus* ticks in a tularemic focus, in 1949, and which later had been maintained in the laboratory by means of passage through animals and ixodial ticks. It was established by repeated checking that this strain had maintained a high degree of virulence during the 5-year period.

The diagnosis of tularemia in the animals which died was established according to the lapse of time until death characteristic of this infection, the pathological changes in the organs of the animals, the presence of tularemia bacteria in smears of organs and tissues, and the isolation of the organism on solid egg-yolk medium.

In the experiments of transmitting the infection by the nymphs we used chiefly common field voles, and some lemmings and white mice; for transmission by the sexually-mature ticks guinea pigs were used as the recipient animals.

The larvae of both species, after sucking their fill, fell off the animals on the second to fifth day after being attached. On the average, the satiation of the larvae lasted 3 days. By means of repeated 2- and 3-time attachment of the larvae to the infected animal, at intervals of a day between the attachments, we managed to time the falling off of some of the satiated larvae to the last day of the animal's life, that is to its agonal period, and in this way obtain the most highly infected larvae. This fact was established by the presence of a large number (3 to 4 "balls"*) of bacteria in the smears from the larvae, and by biological investigations.

* A scale of points system of estimating numbers of bacteria, the equivalent of which apparently does not exist in the English language. (Translator's note.)

Experiments with Dermacentor marginatus *Ticks*

For the purpose of clarifying the possibility of transmitting the infection by way of a single nymph, experiments were performed on 41 common field voles and 8 white mice, a total of 49 animals. The nymphs were dropped onto the animals 6 to 19 days after molting from the larval stage and 11 to 26 days after having been infected by the donor animal. Forty-three nymphs sucked their fill on 37 voles and 6 white mice, and fell off satiated; no nymphs were found on the other 4 voles and on 2 white mice. At least 4 of the 6 nymphs had been eaten by these 6 animals because 4 of them (2 voles and 2 white mice), died of tularemia. The other 2 nymphs possibly died without having sucked and were lost in the litter.

Of the 43 animals on which a single nymph apiece had fed, 11 animals (10 voles and 1 white mouse) died of tularemia in 8 to 16 days. Pathoanatomical changes typical of tularemia were found in organs of all dead animals; their spleens and blood showed tremendous numbers of tularemia organisms, and cultures from their organs produced a profuse growth of the bacteria.

Two voles died of other causes. The other 30 animals survived without showing any signs of disease.

Of the 43 nymphs which had fallen off the animals we checked 30 biologically (by passage through white mice) for presence of the infection. Twenty-seven nymphs were found to be infected, while in 3 nymphs no infection could be found. Therefore, failure to transmit the infection to some experimental animals is explained by the fact that the nymphs had not been infected, apparently because they had lost the bacteria in the process of molting. Thus, in the process of feeding, the nymphs transmitted the infection to the voles and mice in 27 percent of the cases. It must be supposed that the non-transmittal, in 73 percent of the experiments, was associated with an inadequate degree of infection in the remaining nymphs. Our experiments for determination of the degree of infection in the nymphs showed that the number of tularemia bacteria in the unfed nymphs, before being dropped onto the animals, may range from 10 to 1000.

With the increase in the number of infected nymphs attached to the animals, the number of cases of transmission of tularemia resulting in death of the animals, is increased. Eight or more satiated *D. marginatus* nymphs per animal always resulted in an infection in our experiments.

An investigation was conducted of excreta of well fed infected nymphs. Suspensions of the excreta of each of 2 nymphs were injected subcutaneously into each of 2 white mice. One of the mice died of tularemia; the other mouse was not infected. The feces were examined on the day of their excretion. From suspensions of 15 well fed infected nymphs (out of the 30 nymphs used for the biological tests) separate cultures were made on solid egg-yolk medium. The nymphs were first washed in 96 percent alcohol (translator's note: 96° indicated in original) for 3 to 5 seconds and then 3 times in physiological solution. The suspensions were prepared by grinding the ticks in a mortar with the addition of 1 or 2 drops of physiological solution. After 1 to 3 days of incubation pure cultures of tularemia bacteria were obtained from 12 nymphs. Of the remaining three, 2 were found to be contaminated with foreign microflora, and 1 remained sterile.

N. G. Olsuf'yev and N. S. Ya[illegible] (1955) were the first to report the possibility of isolating tularemia bacteria by direct culture from ticks; they investigated sexually mature *D. marginatus* ticks collected in their natural habitat.

Our experimental data confirm, therefore, the possibility of isolating pure cultures by direct planting from ticks, in the given case from well fed *D. marginatus* nymphs.

Experiments for transmission of infection by sexually mature *D. marginatus* ticks were performed on 8 guinea pigs. The sexually-mature ticks came from larvae which had been infected by 2 donor animals (hamsters). Prior to this, the nymphs which came from these larvae were attached in groups of 10 to 50 onto 4 field mice, 3 guinea pigs, and 3 rabbits. All the field mice were infected with tularemia, recovered, and acquired immunity; 2 guinea pigs died of tularemia; 1 guinea pig and the rabbit were not infected. Seventy-five to 212 days from the beginning of infection of the larvae, the sexually-mature ticks were attached onto 8 guinea pigs in these experiments. Of these 8, three died of tularemia; in one of these transmission of the infection was observed after the feeding of a single female tick; this guinea pig died of typical tularemia on the 13th day after attachment of the tick; in the remaining 2 cases of transmission of the infection, 6 ticks (3 males and 3 females) had fed on one, and 7 ticks (all males) on the other guinea pig. Five of the 8 guinea pigs were not infected, but, as shown by subsequent biological checking, the ticks which had fed on them (1 male and 4 females) were free of infection.

Fresh excreta from infected, sexually-mature ticks were the subject of another investigation. A white mouse which was inoculated with this excreta died of typical tularemia on the ninth day. These data confirm the observations made by D. A. Golov, V. N. Fedorov, and R. Ya. Chernina concerning the infectivity of the *D. marginatus* tick feces. Another portion of the tick excreta was kept at room temperature for 9 days and then examined biologically; the result of this experiment was negative: the white mouse was not infected.

For the purpose of determining the number of bacteria in the ticks, we used the biological method of examination on white mice; this method has been described in detail in our previous work (V. G. Petrov and N. G. Olsuf'yev, 1953*). We examined satiated larvae by this method 4 times; unfed nymphs, twice; satiated nymphs, 3 times; unfed sexually-mature ticks, twice; and satiated females, once. For repeat examinations we used ticks of different series which were infected from various animals.

We examined the ticks in groups of several units (no less than 5 and no more than 20), which made it possible to obtain average infectivity figures. The satiated sexually-mature (female) ticks, in view of their limited numbers, were examined in groups of only 2 specimens each.

In the larvae which had fallen from a rodent sick with tularemia on the last day of its life we found from 1 million to 10 million bacteria, but in 1 case only 10,000 bacteria were found. After molting into the nymph stage their number of bacteria dropped sharply. We found a maximum of 1,000 bacteria in the unfed nymphs; in some cases the nymphs became sterile.

The lack of uniformity of infection in the nymphs is explained, apparently, by

* Translation at Crerar Library.

the fact that, first, not all the larvae acquire the same number of bacteria when feeding on a sick animal, and, second, there are different degrees of individual parasite resistance to the bacteria during the metamorphosis of the larva to the nymph.

In the nymphs which remained infected, active multiplication of the bacteria occurred during feeding; after they fell from the animals we found from 1 million to 10 million bacteria in each. After molting of the nymph to the imago there was again a decrease in the number of bacteria to 10,000 to 100,000, and a subsequent increase in the number after the ticks fed on the animals: in satiated female ticks we found 1 billion bacteria.

The data obtained concerning growth of tularemia bacteria in bodies of *D. marginatus* ticks during their metamorphosis are fundamentally in agreement with the data from our experiments on *D. pictus* ticks (V. G. Petrov and N. G. Olsuf'yev, 1953).

For the purpose of clarifying the effect of tularemia bacteria on the bodies of ticks, we studied the survival rate of infected and non-infected ticks throughout metamorphosis. The observations were begun on 2 groups of larvae (there were 154 in the first and 85 larvae in the second, which had fallen off the donor animals —common field voles—on the last day of the disease). One hundred larvae, fed on a healthy common field vole, served as controls for both groups. The conditions under which the experimental and control ticks were kept and fed were the same.

In the first experimental group 97 individuals hatched out of the larvae as nymphs, that is, 63 percent. They were all dropped onto animals, but only 18 nymphs (18 percent) sucked their fill, and a total of only 2 individuals (11 percent) became sexually-mature ticks. The latter did not suck when dropped onto animals, and died. Therefore, only 1.3 percent of the individuals in this group of satiated larvae lived to become sexually-mature ticks, but they were not viable and could not suck blood.

In the second group, 84 ticks (100 percent) hatched from the larvae as nymphs; 56 nymphs (66 percent) sucked their fill on the animals; of these, 22 individuals (39 percent) became sexually-mature ticks, and of the latter 12 ticks sucked their fill on the animals. In summary, in this group of satiated larvae 26 percent of the individuals lived to become sexually-mature ticks, but only half of them were viable and could suck blood. The difference between the survival rates of the ticks of the first and second groups depended on the degree of their infection. Bacteriological examination of larvae smears showed that the first group of larvae was infected with bacteria to the value of 4 "balls"; the second, to the value of 3 "balls."

In the control (non-infected) group all 100 larvae became nymphs; 98 nymphs sucked their fill on the animals; they all molted into sexually-mature ticks; 97 sexually-mature ticks of this group (1 male died) were attached to the animals, and 93 individuals sucked their fill.

Thus, 98 percent of the individuals in the control group of satiated larvae lived to become sexually-mature ticks; of these, 93 percent of the ticks were completely viable and capable of sucking blood.

Experiments on transmission of infection with interrupted feeding of ticks were

performed on 3 common field voles. Ten partly fed larvae, taken from a vole which had died of tularemia, were attached to each of them. The transfer of the larvae was accomplished immediately after the animal died. After 2 or 3 days, 4 larvae which had sucked on it fell off one of the voles; the others were not found. This vole died of tularemia on the seventh day after the larvae had been dropped onto it. The diagnosis was confirmed by isolation of a culture of tularemia bacteria from the animal's spleen. Two and 3 larvae, respectively, sucked on the other 2 voles, but the animals were not infected.

Experiments with Rhipicephalus rossicus *Ticks*

Experiments on the transmission of tularemia via infected nymph were performed on 12 common field voles and 2 steppe lemmings. The interval between the infection of the larvae and dropping of the nymphs onto the animals was 29 to 43 days. Eleven nymphs which had sucked their fill were collected from 14 animals; no nymphs were found on 3 animals. One of these died of tularemia on the seventh day; no nymph was found on it (apparently, it had been eaten). The other animals survived. The 11 nymphs which had fallen off were investigated by the biological method (passage in white mice), and infection was found in 9. Eight nymphs, in which the biological test was positive, were investigated at the same time by culture grown on coagulated egg-yolk medium. Growth of tularemia bacteria in pure culture was obtained in 7 cases; the culture from 1 nymph was found to be contaminated with foreign microflora.

When the number of attached nymphs was increased to 3 to 5 individuals per animal, there was an increase in the frequency of deaths from tularemia; when 10 or more nymphs were dropped onto each animal, transmission of the infection occurred in almost all cases. Positive results of experiments on transmission of the infection by nymphs were obtained on the common field voles, steppe lemmings, and guinea pigs.

In experiments on the transmission of tularemia by sexually-mature ticks 13 guinea pigs were utilized as recipients. The sexually mature ticks were attached 67 to 201 days after they had been infected in the larval phase. (The nymphs which hatched out of the infected larvae were attached in groups of from 10 to 40 individuals onto 16 recipient animals (1 vole, 13 lemmings, and 2 guinea pigs). Of these, 12 animals died of tularemia, and 3 lemmings and 1 guinea pig, on which 62 nymphs had fed, were not infected.) One guinea pig, to which only 1 female tick had been attached (the latter sucked its fill and fell off), died of tularemia on the 13th day after beginning of the experiment. Ten guinea pigs died of tularemia on the 12th to 21st day after attachment of the ticks. From 2 to 8 ticks (males and females) had fed on each of them. Included in this group of guinea pigs was one which died after 2 female ticks had fed on it. These ticks had come from nymphs which had not transmitted the infection to a lemming on which they fed. The diagnosis of tularemia in the guinea pigs was confirmed by isolation of the organism on culture. The other 2 guinea pigs were not infected . The ticks collected from them proved to be free of tularemic infection: subcutaneous injection of the suspension from these ticks did not cause death in white mice.

The feces collected from the infected female ticks contained tularemia bacteria, this was established biologically. The excreta were examined when fresh.

Investigations of growth of tularemia bacteria in *Rh. rossicus* ticks in the course

of their metamorphosis were not complete because of a shortage of sexually-mature ticks. However, the data collected supported the view of non-uniformity of bacterial growth during the different stages of development of the larvae and of the nymphs.

We investigated the larvae which had sucked their fill, and the unfed nymphs, in groups of 10; the nymphs which had sucked their fill in groups of 5. Each group of ticks was checked on mice once.

In the infected larvae we found 1 million bacteria; in the unfed nymphs which had just molted, 100,000; in the satiated nymphs—100 million bacteria.

Separate examination of 2 satiated *Rh. rossicus* females from another series showed that 1 contained 10 million, and the other 100 million bacteria.

The death rate of infected *Rh. rossicus* as well as of the *D. marginatus* ticks in the course of their metamorphosis was greater than that of the uninfected ticks. The observations were begun on 72 larvae which had fallen off a common field vole on the last day of its illness. One hundred and eight larvae which had been fed on a healthy vole served as controls for this group of infected larvae. The conditions under which the experimental and control groups of ticks were kept and consequently fed were the same. In the experimental group 50 larvae hatched into nymphs, that is 69 percent. All these nymphs were attached to animals, but only 10 nymphs (20 percent) sucked to satiation; of these, a total of 4 males and females (40 percent) became sexually mature ticks. When the latter were dropped onto animals only 1 male sucked its fill; the other 3 ticks died. Hence, only 1.4 percent of the individuals of the group of satiated larvae survived to become satiated sexually mature ticks.

In the control group, of 108 satiated larvae 99 nymphs were produced (92 percent); of these, 95 nymphs (98 percent) sucked to satiation and 94 (99 percent) became sexually mature ticks. The latter were dropped onto animals, sucked, and 65 males and females (69 percent) sucked until satiated. In sum, 60 percent of the satiated larvae survived to become satiated sexually mature ticks.

Just as in the experiments with *D. marginatus,* we checked the possibility of transmission of the infection to *Rh. rossicus* larvae in interrupted feeding. The incompletely fed larvae, collected from a vole which died of tularemia, were attached to 4 white mice and 1 vole, on the same day, in groups of from 3 to 10 larvae per animal. Only a single satiated larvae fell off 1 white mouse on which 10 larvae had been placed; no larvae were found on the other 3 white mice and the vole. Three white mice died of tularemia. Three, 5 and 10 larvae had been attached to them, respectively. Apparently, the mice had eaten these larvae, because no satiated ticks were found on them. The time of death of the mice after attachment of the larvae ranged from 6 to 8 days. One white mouse and the field vole survived without showing any signs of disease.

CONCLUSIONS

1. *Dermacentor marginatus* and *Rhipicephalus rossicus* ticks which had been infected in the larval stage are capable of transmitting the tularemic infection to susceptible animals also in their subsequent stages of development. The transmission may be accomplished by a single nymph or by a single sexually-mature tick. Larvae of both species of ticks can also transmit the infection from a sick to a healthy animal by means of interrupted feeding.

2. Transmission of the infection by ticks occurs both by means of feeding on an animal and through the latter's eating the infected ticks. The transmission of infection is also possible through tick excreta.

3. Proliferation of tularemia bacteria in *D. marginatus* and *Rh. rossicus* ticks is not uniform in the course of their metamorphosis. Feeding of the ticks (nymphs or sexually-mature individuals) is accompanied by an increase in the number of bacteria; the process of molting, by a decrease; and, when they are not adequately infected, individual ticks may become entirely free of bacteria during the period of molting from one stage into the next (particularly, from larva to nymph).

4. *D. marginatus* and *Rh. rossicus* ticks are not indifferent to infection with tularemia bacteria. The greatest mortality in both species is observed in the nymph stage, when vulnerability of the ticks increases with the degree of their infection.

5. Satisfactory results of bacteriological examination have been obtained by means of direct cultures of suspensions of infected satiated nymphs of *D. marginatus* and *Rh. rossicus* ticks on coagulated egg-yolk medium.

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PENTASTOMIDA FROM REPTILES OF LAN YÜ ISLAND (TAIWAN), WITH A DESCRIPTION OF *RAILLIETIELLA HEBITIHAMATA* N. SP.*

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AND
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U. S. Naval Medical Research Unit No. 2, Taipei, Taiwan

In March 1959, as a part of other parasitological investigations, a survey was made of the Pentastomida from the reptiles of Lan Yü, a small island in the Pacific Ocean 45 miles east of the southern tip of Taiwan. It is currently recognized as Lan Yü (Chinese) and Orchid Island (designation by westerners). In the earlier literature the island was listed as Ko-To-Shu (Japanese) and Botel Tobago.

MATERIALS AND METHODS

All hosts were taken alive or were killed in the field just a few hours prior to examination. Each organ system was examined separately under a dissecting microscope. Pentastomids were killed in hot water then fixed immediately in FAA (formalin-acetic acid-alcohol). After 12 to 15 hours of fixation, specimens were washed and stored in 70 percent alcohol plus 2 percent glycerine and later studied without staining. All measurements are reported in millimeters.

PENTASTOMIDA IN LAN YÜ COLLECTION

Raillietiella orientalis (Hett) was recovered from the body cavity and lung of the snake *Elaphe carinata* (Guenther), the lung of the snake *Psammodynastes pulverulentus* (Boie), and *Kiricephalus pattoni* (Stephens) was recovered from the lung of *E. carinata*. *R. geckonis* (Diesing) Sambon was collected from *P. pulverulentus*.

Raillietiella orientalis has not been previously reported from *E. carinata*, but has been taken from *Elaphe quatuorlineatus* Lacont (Heymans 1935). *K. pattoni* has not been reported previously from *E. carinata*.

In addition to the above collections, a new species of pentastomid was recovered and is here described.

Raillietiella hebitihamata n. sp.
(Figs. 1, 2, 3, and 4)

Characteristics of family Cephalobaenidae.

Head (plate I, fig. 1) roughly triangular; anterior end distinctly snoutlike. Dorso-lateral papillae well defined. Anterior ones project dorsally from level of the anterior hooks. Anterior hooks fang-like and without accessory spines (fig. 3). Posterior hooks much larger than the anterior ones, without accessory spines, markedly blunt, and usually withdrawn into integumentary coverings (fig. 2). Lobi parapodiales present but not prominent at both anterior and pos-

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* The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of the Navy.

We are indebted to Dr. Robert F. Inger, Curator of Reptiles, Chicago Natural History Museum for identification of hosts and to G. M. Malakatis HM 1, USN, and James E. Reese, HM 1, USN, Parasitology Department, NAMRU No. 2, for technical assistance in examining hosts.

terior hooks. Segmentation often indistinct but marked by slight, intersegmental elevations. Terminal abdominal papillae blunt and pointing sharply laterad (fig. 4). Mouth opening triangular with apex posterior, and supported by sclerotized plates.

Type Host: *Hemidactylus frenatus* Duméril and Bibron.

Organ: Lung.

Locality: Lan Yü Island (known also as Ko-To-Shu, Botel Tobago and Orchid Island).

Holotype: Female, U. S. Nat. Mus. Helm. Coll. No. 39108.

Allotype: Male, U. S. Nat. Mus. Helm. Coll. No. 39109.

Female: Length 13, no. of segments 29 plus head and terminal segment. Width of head at anterior tip 0.28; at level of anterior hooks 0.55; at level of posterior hooks 1.0; width of body at widest point 1.0. Length of anterior hooks from base to tip 0.1; length of posterior hooks from base to tip 0.37; width of mouth at base 0.1; length of mouth 0.13. Breadth of caudal papillae 0.10. Genital pore at base of abdomen. Eggs oval, 0.089 × 0.068.

Male: Length 5.0; no. of segments 29 plus head and terminal segment. Width of head at anterior tip 0.20; at level of anterior hooks 0.35; at level of posterior hooks 0.75; width of body at widest point 1.2. Length of anterior hooks from tip to base 0.059; length of posterior hooks from tip to base 0.16; breadth of terminal papillae 0.096. Genital pore at base of abdomen.

This species differs from others described for the genus *Raillietiella* by the peculiarly blunt posterior hooks, (fig. 2). All others of the genus possess remarkably sharp, fang-like hooks making this character specific.

R. hebitihamata was also collected from the lungs of *Japalura swinhonis* (Gunther), *Mabuya longicaudata* (Hallowell), *Sphenomorphus indicus* (Gray), and *Gecko monarchus* (Duméril and Bibron).

DISCUSSION

The several known species of this genus occur in both snakes and lizards, and Gedoelst (1921) reported *R. indica* Ged. from *Bufo*. Except for the latter, all of the hosts recorded for this species of the genus are snakes which feed generally on mammals or other reptiles, geckos (*Gecko*) which feed primarily on insects, or lizards such as *Varanus* which feed on larger crustaceans, fishes, amphibians, birds, and mammals. Obviously this group of Pentastomida has become adapted to a remarkably wide variety of hosts which have a comparable variety of feeding habits. This situation is re-emphasized by the occurrence of this newly-described species in 6 species of hosts. One is at a loss to make any postulation as to their life cycles and indeed may wonder if *Raillietiella* always employs an alternate host. In any event there is no pattern of food habits on the part of its hosts which suggest a common pattern in life cycles.

As has been previously stated (Self aand Kuntz, 1957), several species of *Raillietiella* presently recognized are so closely related morphologically as to defy certainty of identification in many cases. The blunt posterior hooks in the species described here is more distinctive than the definitive characters of most other species. It conforms most closely with the characteristics of group one of Heymans (1935).

SUMMARY

Raillietiella orientalis was recovered from *Elaphe carinata* and *Psammodynastes pulverulentus*. *Kiricephalus pattoni* was taken from *E. carinata*. All are new host records.

In addition *Raillietiella hebitihamata* n. sp. is described from *Hemidactylus frenatus*. It also occurs in *J. swinhonis, H. frenatus, M. longicaudata, S. indicus* and *Gecko monarchis*.

This paper re-emphasizes the wide variety of hosts, in terms of feeding habits, parasitized by members of the genus *Raillietiella*. Their life cycles can hardly

conform to the patterns shown by other pentastomida.

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DESCRIPTION OF PLATE I

All drawings were done with the aid of a camera lucida.

FIGURE 1. Slightly oblique view of head of female showing the snout shape of the tip of the head, the triangular shape of the mouth with supporting plates and the arrangement of the hooks.

FIGURE 2. The posterior hook showing the characteristic blunt point and its relationship to the fulcrum.

FIGURE 3. The anterior hook showing the characteristic pointed shape and relationship to the fulcrum.

FIGURE 4. The posterior end of the female showing the terminal papillae.

ANNOUNCEMENT

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The American Institute of Biological Sciences is currently translating and publishing seven Russian research journals in biology. These journals are translated with support from the National Science Foundation, which is eager that such information be more widely distributed to biologists throughout the world. It is hoped that this material will aid biologists in research, prevent duplication of work, give some idea of the work being done by Soviet scientists in the field of biology, and also bring about a better international understanding among scientists.

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The journals currently being translated are: Doklady: Biological Sciences Section; Doklady: Botanical Sciences Section; Doklady: Biochemistry Section; Plant Physiology; Microbiology; Soviet Soil Science; and Entomological Review.

In addition to its program of Russian Biological Journal translations, the AIBS has instituted a separate program of translation and publication of selected Russian Monographs in biology.

It was felt that the program of Journal translations was not sufficient to cover all of the significant work being done in all fields of biology by Russian scientists. With the aid of competent authorities, the AIBS has translated and published six Russian monographs and one monograph is in the process of being published. In addition, several prominent monographs in various biological areas are being considered by the AIBS and the National Science Foundation for translation and publication. The monographs that have been published are: Origins of Angiospermous Plants by A. L. Takhtajan; Problems in the Classification of Antagonists of Actinomycetes by G. F. Gauze; Marine Biology, Trudi Institute of Oceanology, Vol. XX, edited by B. N. Nikitin; Arachnoidea by A. A. Zakhvatkin; and Arachnida by B. I. Pomerantzev. The manuscript for Plants and X rays by L. P. Breslavets is in the final stages of preparation and should be published early in 1960.

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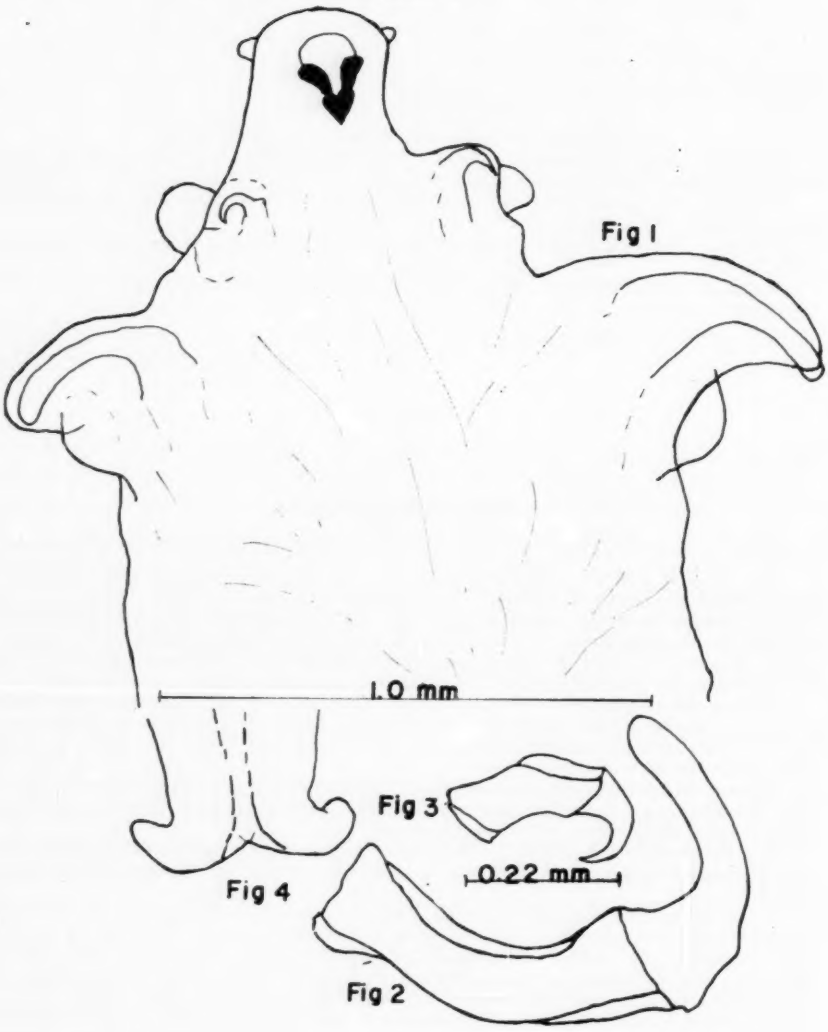
PLATE I



RESEARCH NOTE

PARATHELPHUSA CEYLONENSIS C. H. FERN., SECOND INTERMEDIATE HOST OF *PLEUROGENOIDES SITAPURII* (SRIVASTAVA).

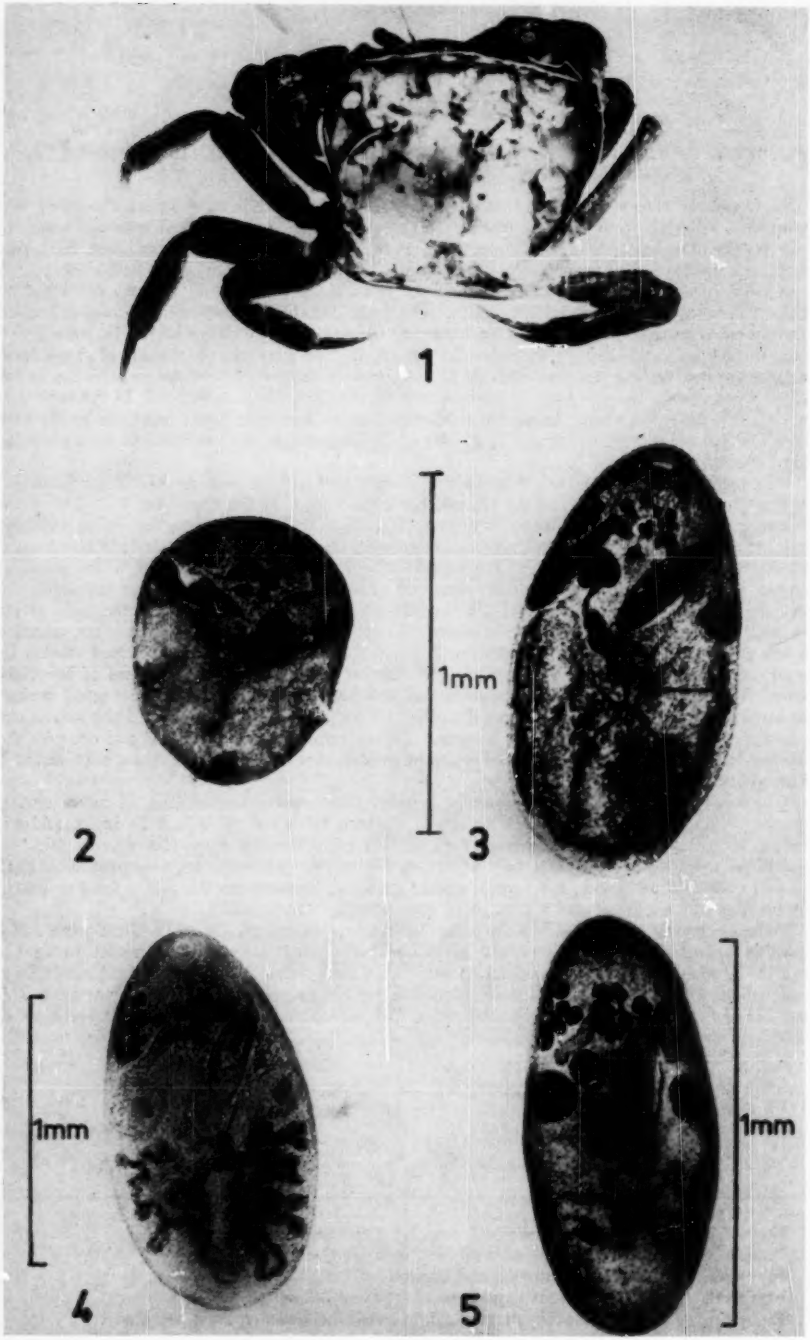
In December 1958 we reported that over 90 percent of the fresh-water crabs, *Parathelphusa ceylonensis*, collected from a paddy field in Nugegoda, Ceylon, were infected with a progenetic metacercaria (Dissanaike and Fernando, 1958, Proc. 14th Ann. Sess. Cey. As. Adv. Sci., Abstracts, pp. 43–44). Its morphology suggested that it was a lecithodendriid belonging to the genus *Pleurogenoides*. Metacercariae fed to the frog, *Rana hexadactyla* Lesson, developed to adult worms in the duodenum within 3 days. We made certain that they were worms developed from the metacercariae that were fed by following the day-to-day changes of the juvenile flukes in the duodenum until they showed eggs in the uteri. We also examined control frogs from the same stock. The species proved to be *Pleurogenoides sitapurii* (Srivastava) which was described from *Rana cyanophlyctis* in India (Srivastava, 1934, Bull. Acad. Sci. U.P. Agra and Oudh **3**: 239–256). We have found the trematode also in that host taken from the paddy field where infected crabs were collected. A species of *Pleurogenoides* has not hitherto been recorded from Ceylon.

The metacercarial cysts are 0.5 to 0.8 mm in diameter and are seen as whitish or brownish spherical bodies in the muscles of the cephalothorax and limbs of the crab (fig. 1). The metacercaria is progenetic (fig. 2) and protandrous. Its salient features are seen best in an extended specimen (fig. 3). The body is uniformly covered with fine spines. The suckers are about equal with the oral sucker subterminal and the ventral sucker more or less equatorial. The muscular pharynx is followed by a distinct oesophagus and short, divergent intestinal ceca which do not reach the level of the ventral sucker. The genital pore is on the left margin of the body at the level of the pharynx. The cirrus sac is large and extends almost to the ventral sucker, crossing the left cecum. The testes are rounded or ovoid, the left being smaller and pushed caudad by the cirrus sac. The ovary is intercecal to the right of the mid-line, near the end of the right cecum. The receptaculum seminis is oblique and lies between the ovary and the ventral sucker. The vitellaria are asymmetrical and consist of 6 to 8 follicles each. The coils of the uterus can be distinguished although no eggs are present. The excretory vesicle is bilobed and occupies the posterior half of the metacercaria, pushing the other structures cephalad. It opens subterminally on the ventral surface.

The adults obtained from the naturally infected *Rana cyanophlyctis* (fig. 5) agree closely with the original description of *Pleurogenoides sitapurii* from the same host in India, and are identical with the adults recovered from the experimentally infected *Rana hexadactyla* (fig. 4). They differ from the metacercaria only in having the uterus filled with eggs measuring 0.025 to 0.03 mm × 0.012 to 0.015 mm, the ventral sucker distinctly post-equatorial, and a smaller excretory bladder which is V-shaped with a short stem opening subterminally.

A large number of snails from the same habitat was examined. Most of them were *Indoplanorbis exustus* from which emerged xiphidiocercariae that we strongly suspect to be the larva of *P. sitapurii*. We are grateful to Dr. Yeh Liang-Sheng, Department of Parasitology, London School of Hygiene and Tropical Medicine, for his suggestions.—A. S. DISSANAIKE, *Department of Parasitology, University of Ceylon, Colombo,* and C. H. FERNANDO, *Department of Zoology, University of Malaya, Singapore.*

FIGURE 1. Dissected crab with metacercariae indicated by arrows.
FIGURE 2. Metacercaria soon after liberation from cyst (stained).
FIGURE 3. Metacercaria flattened and stained.
FIGURE 4. Adult worm from experimental frog (unstained).
FIGURE 5. Adult worm from naturally infected *Rana cyanophlyctis* (stained).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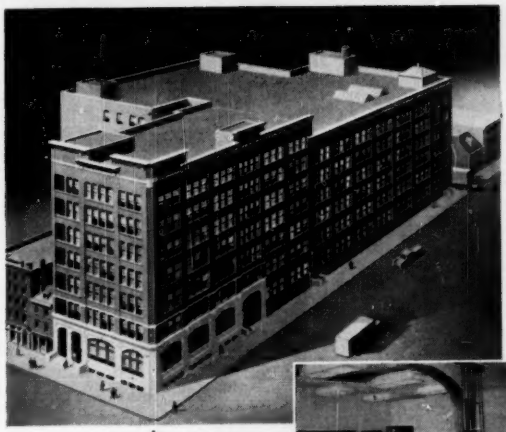

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Telorchis bonnerensis N. SP. (TREMATODA: DIGENEA) FROM THE INTESTINE OF LARVAL *Ambystoma macrodactylum* BAIRD, FROM NORTHERN IDAHO.
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